

Towards an election disappointment

Britain's forthcoming election will not solve the problems that matter most, notably the incorrigible waywardness of British governments between their occasional encounters with their electorate.

ONLY a few months ago, Mr Neil Kinnock, the leader of the British Labour Party, was complaining that an incumbent British government's freedom to call a general election whenever it chooses gives it such an unfair tactical advantage that he had been persuaded in favour of fixed-term parliaments. As if to prove Kinnock's premise false, the present government has now thrown away the supposed tactical advantage of surprise by pre-election electioneering, giving general credence to the belief that there will be a general election on 9 April, more than three months before the government's spell in office comes constitutionally to an end. Although the date has not been fixed officially, the government's electoral prospects would probably be damaged if it settled for another postponement. Something will have to be said this week.

British voters will get no advice from *Nature* on how to cast their votes, but they will be offered such information as is likely also to interest people elsewhere about the electioneers' plans for British science and research. Meanwhile, there is a more general issue of wide interest. The coming election, likely (judging by the opinion polls) to produce a close result, is in a deep sense irrelevant to the British problem. For the British, who have long prided themselves on having had a hand in the origin of democracy (and who were fond of calling the British Parliament 'the mother of parliaments' until Mr Saddam Hussein debased the form of words) are badly governed and are likely to remain so, whatever the outcome of the election.

What has happened to British science and research over recent decades (and not just the tenure of the present government) provides ample illustration. First, there have been successive organizational changes (coupled with the names of Trend, in the early 1960s, and Rothschild in the early 1970s) on which neither the scientific community nor the wider body politic has been adequately consulted. The wisdom of the more recent transformation of the defence research laboratories into a free-standing agency, with all its implications for the careers of able researchers, has been virtually decreed without discussion.

Second, successive governments have thought fit to make arbitrary budget changes (usually cuts) without apparent care for the consequences; the reduction at the end of 1990 of university budgets by 9 per cent (13.5 per cent after allowing for changes in the treatment of foreign students) began the present phase of rot.

Third, British governments repeatedly tell the research

community that it must manage its own affairs more effectively — and then turn round and tell it that it cannot. British contributions (and notably their currency fluctuation) to the European Laboratory for Particle Physics (CERN) at Geneva have been contentious since 1970, with governments insisting that they must be a charge on the 'science budget'. But when (in 1984) the scientific community sets up a committee to consider opting out, the government insists that international considerations are paramount, and reserves the decision for itself.

But research is not the only part of British public life to have been played with in this way. Ill-considered and arbitrary decisions are forever filtering out of Whitehall (the collective name for the mostly ungainly ministry buildings scattered through London, Edinburgh, Cardiff and Belfast). Educational interests have been especially hard-hit: the laudable creation of a national curriculum has been accompanied by such confusion over school-leaving qualifications that young people now entering secondary schools cannot know what qualifications they can hope for when they leave (if they stay for more than four years). The government's bill requiring schools to appoint their own inspectors (hitherto the civil servant invigilators of educational quality) from the private sector was defeated in an ambush in the House of Lords, and would otherwise have become law before the election.

Some ineptitudes of British governments are almost laughable, such as the decision to will the Channel Tunnel as a rail link with France, but not, yet, to connect it to the British rail network. Others are not laughable at all; indecisiveness on the regulation of financial services (on which the British Parliament has spent many weeks in the past decade) has allowed financial scandals to proliferate, while the most likely consequence of chopping and changing on broadcasting policy is a general debasement of quality.

The underlying problem is that Britain is a democracy only in the sense that Members of Parliament (or at least of the House of Commons) are elected. British governments are elective dictatorships. That description, first used by Lord Hailsham (hardly a constitutional radical) explains both the waywardness of governments' day-to-day management of affairs and the polarization of the political parties, who see it as their first duty to undo the damage done by their opponents while in office. In advance of this year's election, there has been much talk



of electoral reform, but the real need is for some of the checks and balances that would give those elected, by whatever means, power to moderate what governments propose. □

American free trade

The North American Free Trade Agreement deserves better than the back-burner until the US election.

It is a shame that one of the casualties of the industrial world's almost general election fever is that the US Administration seems, at least for the time being, to have put on ice the North American Free Trade Agreement, the plan to extend the benefits of the 1990 free trade agreement with Canada to Mexico as well. Although the negotiations are believed to be well advanced, Mr Brian Mulroney, the Canadian Prime Minister, said at the weekend that he did not expect the deal finally to be struck until the "end of the year", which means after the US election.

The difficulty seems to be that this election campaign is not the best background for a trade deal; voters worried about jobs are not convinced that free trade is not a zero-sum game, and the administration reflects that. Even the bilateral agreement with Canada is in trouble, with corrosive complaints that Canadian lumber is too cheap (because of arrangements for financing the cost of removing dead tree-stumps) and that some Japanese cars manufactured in Canada do not satisfy the requirement that half their value should derive from North America (even though the engine blocks are actually cast in the United States, and imported from there). The extension of the agreement to include Mexico, even with reservations about the speed at which tariffs would disappear, has raised more worrying hackles in the United States. Some say it is a way of exporting jobs south of the border, others that it is a device for exporting pollution (to a place where regulatory standards are less cramping).

Inevitably, arguments such as these, whether soundly based or not, are readily amplified in election years. Meanwhile, the economic benefits likely to be won by the United States itself from a larger market are admittedly more distant and therefore seem less tangible. But there are political and cultural benefits as well, not the least of them deriving from Mexico's potential role as a bridge between the United States and the still imperfectly comprehended territory further south. It is understandable that President George Bush should, for the time being, let the hustings make the conclusion of an agreement seem less urgent, but is it forgivable? □

Retroviral Michelangelo

What will epidemiologists make of the scare that the world's hard discs would be wiped clean last Friday?

MICHELANGELO's 517th birthday has come and gone without bringing computer-dependent parts of the world to a

halt. Although the hard discs of personal computers at various scattered locations were wiped free of data by the computer virus designed to become active on that day, most machines appear to have survived intact. Epidemiologists will probably find that actual outbreaks of computer disease are too few and far between to be informative in the technical sense. Certainly there is only a small chance that the origin of this well-publicized outbreak can be identified from what is known of the incidence of outbreaks last Friday.

Luckily, some of the imponderables that usually have epidemiologists scratching their heads when confronted with a novel infection will not on this occasion be impediments to understanding. For example, the sections of the personal computer population at risk from infection are known in advance: computer viruses subvert a computer's operating system just as a real virus subverts the genetic apparatus of a cell, so that the feasibility of successful infection is, for the time being, necessarily specific to computers with the vulnerable system. (It may be different when 'open' systems, allowing intercommunication between computers, are a reality.) Similarly, the mechanism of transmission is not, in this case, in doubt: hard discs are infected by corrupted floppy discs, and then themselves generate infected floppy discs (verifying Koch's postulate).

It is also relevant that the Michelangelo virus resembles a retroviral infection, say by HIV. It could hardly be otherwise. Computer viruses corrupting hard discs the instant they infect them would not get very far in the world. This is why quickly lethal virus infection, as Marburg virus or that responsible for Lassa fever, are not such serious public health problems as, say, that for measles. The designers of Michelangelo were clearly aware that their virus should have a long latency period, allowing plenty of opportunity for cryptic replication as pro-virus rather than as virus proper. On this occasion, the long latency and also the advance warning allowing computer users to take simple prophylactic measures (such as resetting system dates) will also impede attempts to track down the focus of infection.

By now, the whole world knows what kinds of people are likely to be responsible. They are teenage computer enthusiasts who, a few years ago, were busily hacking their way into the Pentagon's databases, but who have now seized new opportunities for causing global chaos. From time to time, some of them are caught by the police and given suspended jail sentences. But before all innocent teenagers smart enough to use assembly language are picked up for questioning, it would be wise to remember that the Michelangelo virus is the best thing ever to have happened to software manufacturers seeking to protect their copyrights. The past week's fuss has also been a shot in the arm for those who sell software designed to neutralize known computer viruses. The real culprit may be a grown-up. □

Japanese companies trim spending on research

- Recession halts five years of rapid growth
- Capital expenditures suffer sharpest cuts

Tokyo

MAJOR industries in Japan have begun to freeze, or even to reduce, what they spend on research and development (R&D). The cutbacks suggest that the global recession that has so dominated Western economies has begun to reach Asian shores as well.

News that a five-year spending spree has come to an end is expected later this month when companies reveal their spending plans for the fiscal year that begins 1 April. It will be a shock to those who think of Japanese industry as an unstoppable juggernaut. Industry has been the driving force in Japan's rise to the top in the share of its gross national product — now about 3 per cent — devoted to R&D. In recent weeks there have even been reports that Japanese industry is spending more on R&D, in absolute terms, than the United States. But Japan's position at number one, even if real, may not last long.

A survey of five of Japan's major electronics companies — Hitachi, Toshiba, Fujitsu, Sony and NEC — shows them holding R&D spending in 1992 to current levels. The *Nihon Keizai Shimbun*, Japan's leading financial newspaper, reported last week that the research budget for a sixth industrial giant, Mitsubishi Electric, may even fall below its 1991 level of ¥190,000 million (\$1,460 million).

These six electronic companies alone spent over ¥1.7 billion (\$13,000 million)

in 1991 and account for well over 15 per cent of all Japanese industrial spending on R&D. Their decision to hold down spending is expected to have a profound effect on overall outlays for industrial R&D.

The same is true of the automobile industry where, for example, Nissan is

cluding those for the final stages of development of products, is being severely trimmed. Most of the major electronics companies, for example, expect to make reductions of 20 to 30 per cent over last year. And 1991 levels showed little or negative growth compared with the boom years of 1988, 1989 and 1990.

The decision to hold down spending is a response to plummeting sales brought about by the recession in the United States and Europe and by rapidly declining growth in the Japanese domestic market as well. In the past few weeks, many companies have revealed that their profits for 1991 will be much lower than expected; the parent company of the Sony group, for example, has gone into the red for the first time in decades.

The cutbacks will be felt most keenly by researchers in the semiconductor industry who are close to the production line. That is particularly true for spending on capital items. The outlook, says a research manager in the microelectronics section of one major electronics company, "gets worse and worse every day". At the same time, the manager says, the "crazy competition to produce next-generation computer chips will continue".

The situation is a little brighter in the basic research laboratories opened by electronics companies during the

boom years of the late 1980s. "It's a difficult situation", says the head of one such laboratory, "but I do not think the reduced level of funding will pull back our research. We will just have to use our brains rather than more money."

Despite curtailed spending, most companies plan to continue their vigorous recruitment of scientists. Japanese industry is expecting a severe shortage of trained engineers and scientists towards the end of this decade as birthrates decline and higher-paying jobs in finance deplete the available pool of talent.

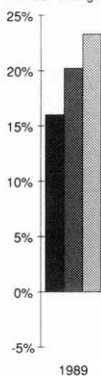
In the face of these cutbacks, government officials remain optimistic about the health of Japan's economy. Officials at the Economic Planning Agency, who monitor the economy, say Japan is not in recession but rather that growth is just "decelerating" to a more stable rate. Even so, it is expected that public works expenditures for fiscal year 1992 will be brought forward to stimulate growth and that the official discount rate will be further reduced. In the meantime, the budget is stalled in the Diet because of yet another financial scandal involving politicians.

David Swinbanks

Research spending takes a nosedive

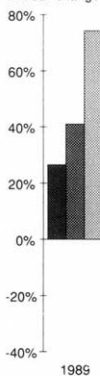
Research & Development

Annual change



Capital Investment

Annual change



■ Hitachi ■ Mitsubishi Electric ■ Showa Denko

*1992 numbers are estimates based on preliminary company information

expected to keep R&D expenditures close to the 1991 level of ¥250,000 million (\$1,920 million). And growth in the chemical and biotechnology industries, which in the West has been fairly resistant to the recession, is also expected to be very small, at most one or two per cent.

Because companies must still contend with rising personnel costs and the effects of inflation, a flat budget means less money for research equipment and facilities. Capital investment in production facilities, in-

TPA ruling shakes up biotech industry

In biotechnology, a looming recession is not the only factor holding down Japanese research and development spending. A legal victory late last year by the US company Genentech in its patent fight over the heart-treatment drug tissue plasminogen activator (TPA) (*Nature* 354, 4; 1991) has forced many Japanese companies to pull back their investments.

The case has taught Japanese companies that you have to be a front-runner to succeed, says Mitsuru Miyata, editor of *Nikkei Biotechnology*, a newsletter that

closely monitors the Japanese industry. He says that many companies that leapt into biotechnology research in the 1980s thought that they could just "follow the pack". Now, he says, they realize it is "no good to be third or fourth".

The declining investment in biotechnology in Japan stands in stark contrast to the situation in the United States, where investment last year soared to a record \$30,000 million. But Miyata expects the Japanese companies that remain after the shakeout will be better able to compete globally. **D.S.**

French-Indian project in jeopardy

New Delhi

A JOINT venture between a French pharmaceuticals company and the Indian government to make an injectable polio vaccine for mass distribution appears to be doomed by the government's decision to support an older, oral vaccine.

Institut Marieux (IM) is building a \$50-million plant outside Delhi to produce the injectable vaccine. Work on the plant, one of the biggest in Asia, began in 1989 after an agreement between the two countries. The plant, which will have the capacity to produce 50 million doses of injectable polio vaccine (IPV), is scheduled to be completed by the end of the year. But it so far has received no orders for its vaccine.

The company is now thinking of withdrawing from the joint venture. "If the health ministry does not want to use our product, we might as well close down the project", says a spokesman. A decision is expected later this month at a board meeting here of the joint venture.

The company is caught in the middle of a dispute between two Indian government agencies. The Indian Health Ministry, although it did not oppose the agreement when it was reached in November 1988, has said more recently that it will follow the recommendation of the World Health Organisation (WHO) and use an oral polio vaccine (OPV) in its national programme to eradicate the disease. As a

result, it has so far refused to place any orders for the vaccine to be manufactured at the IM plant.

Officials from the Indian Department of Biotechnology, which set up the agreement with the French company, are highly embarrassed by the political feud. A spokesman for the department says that it will seek ways to export the injectable vaccine if the health ministry remains opposed to its use in India. The state-owned Indian Petrochemicals Ltd. is the other Indian partner in the joint venture with IM.

The health ministry says it has both scientific and economic reasons for its choice of an oral vaccine. The live oral vaccine confers humoral and intestinal immunity much faster than the injectable version, it says, and extends immunity to a wider population. In addition, the oral vaccine costs one-tenth as much and can be administered with drops to the mouth. In contrast, the injectable vaccine requires trained personnel, needles and syringes, and sterilization equipment. The ministry also rejects as impractical a suggestion to combine the injectable vaccine with the DPT (diphtheria, pertussis and tetanus) vaccines, pointing out that each has a different vaccination schedule.

Last week it became clear that the health ministry has won its battle. A statement issued by the prime minister in Parliament declared that the oral vaccine will

continue to be used until feasibility studies of the injectable vaccine are completed. Those studies, large-scale trials that will take four years to conduct and analyse, are intended to test the idea of combining the polio vaccine with the DPT vaccine. But Marieux officials say that they cannot wait four years before deciding whether to begin production of their vaccine.

The health ministry cites a report from WHO to bolster its case. That document, issued last December, says that "OPV is the only vaccine that can displace circulating wild virus and thus assure community and individual protection". The report says that the IPV is suitable "only in industrialised, polio-free countries with high standards of sanitation and no circulation of wild polio virus, and where vaccine cost is no problem".

Not all Indian polio experts agree with the WHO assessment. Renu Patel, a Bombay paediatrician, calls that view "discriminatory" and sees it as an example of "recommending the latest vaccine to the rich and the oldest one to the poor". Patel has used an injectable vaccine effectively to immunize the children in two Bombay slums. A.B. Desai, a former president of the Indian Academy of Paediatrics, estimates that a campaign using an oral vaccine will require a minimum of ten doses. And he predicts that high drop-out rates will doom such an effort. **K.S. Jayaraman**

TECHNOLOGY TRANSFER

British Technology Group staff win fight for control

London

Management and staff of the British Technology Group (BTG), the world's largest technology transfer organization, have won their battle for control of the company.

Peter Lilley, UK trade and industry secretary, announced last week that the government has chosen a consortium headed by Ian Harvey, existing BTG chief executive, as the preferred future owners of the group. On Monday this week Harvey's team was negotiating with government officials over the terms of the company's sale. The announcement ends a period of uncertainty during which interested consortia were asked to make confidential bids, naming the price and describing their plans.

Harvey said last week that he believed his bid had been successful because of its "long-term aim of enhancing BTG's business in international technology transfer". But the government's decision will also avoid political controversy in the run-up to a closely fought general election.

The other front-runner in the race to win control of BTG was a consortium including BTG's principal international

competitor, the US group Research Corporation Technologies (RCT). The opposition Labour party attacked the Conservative government for allowing a US competitor to bid for control of a group that is expanding into the US technology transfer market, and whose portfolio of patents includes many inventions from British academic researchers (see *Nature* 355, 485; 1992).

Until negotiations with the government are complete, BTG managers are unwilling to name all the members of their consortium, or the exact price they are offering to pay for BTG. Nevertheless, the price is rumoured to be more than £23 million, and Tony Christmas, BTG head of marketing, says: "I think the academic researcher will be pleased with the investors we have lined up".

Researchers have worried that BTG would fall into the hands of short-term investors who lack the vision to transfer technology successfully from academic institutions to industry. None of the investors in the successful consortium will own more than 15 per cent of BTG's shares, and Christmas says that they include a number

of leading British and European academic institutions and research foundations.

John Ashworth, director of the London School of Economics, who headed the RCT-backed consortium bidding for BTG, says he is very disappointed by the government's decision. Although he declined to discuss details of his bid, other sources indicate that the RCT-led consortium was prepared to offer a substantially larger sum than the successful consortium — perhaps approaching £50 million.

Ashworth still believes that there is room for Research Corp. to expand into the UK technology transfer market. "[RCT president] Gary Munsinger and I are talking about the possibility of opening an autonomous business in the United Kingdom," he says. Ashworth says that the business would concentrate on the transfer of technology invented by academic researchers.

Christmas rejects any suggestion that BTG is turning its back on academic inventors. "Let me be quite clear," he says. "99 per cent of our business is pure technology transfer."

Peter Aldhous

Changing of the guard leaves Cray out

Washington

AN escalating shift from traditional supercomputers to massively parallel machines has left the industry's founder, Seymour Cray, with no products, no customers, and chilly prospects for success.

Two years ago, the titans of the supercomputer industry were assuring researchers that although new 'massively parallel' machines might be able to run test programmes with blinding speed, real-world problems required real computers. That means linear or 'vector' processors — the type of machines that have traditionally dominated the industry founded by Cray, who created Cray Research, Inc., in 1972 and then, 17 years later, left to begin Cray Computer Corporation.

Now, those same companies are designing massively parallel machines as fast as they can shift engineers from the traditional models. And Seymour Cray is out in the Minneapolis cold.

In December, after Cray missed a series of deadlines, the Lawrence Livermore National Laboratory cancelled its order for the first Cray 3, the machine on which Cray and his new company had pinned their hopes. Livermore also broke off an agreement to help Cray develop software for the new machine. With no other buyers on the horizon, Cray Computer announced last month that it was halting development of the Cray 3 and would try to produce a smaller and cheaper version of the same machine.

Even at Cray Research Inc., the word is out that the old ways will have to go. After ignoring the upstart massively parallel machines while it perfected the traditional vector processing supercomputers (epitomized by last year's C90 machine), the company has now made a somewhat tardy leap into the parallel world.

Last month, at a supercomputing conference in Paris, Cray Research announced that it was developing a series of massively parallel computers based on a new chip — dubbed 'Alpha' — from Digital Research Corporation. The new Cray computers will combine hundreds of Alpha chips — each one as powerful as the Cray 1, the machine that launched the supercomputer revolution 20 years ago. Officials hope to have by 1997 a machine that operates consistently at speeds at or above a teraflop (one trillion floating point operations per second).

Six of the seven leading supercomputing companies — including IBM, Fujitsu, Hitachi and NEC — are now working on massively parallel machines. They are riding on the coat-tails of Thinking Machines, Inc., the start-up company that introduced the first commercial massively parallel computer in 1986 and surprised nearly everyone by actually making it

work. Four of the five National Science Foundation (NSF) supercomputer centres now have at least one massively parallel machine, and half of those are from Thinking Machines.

This shift is due not so much to the success of massively parallel machines — which still lack sufficient software to solve most problems — as to the inability of traditional supercomputer manufacturers to improve their machines as fast as they once could.

While the massively parallel hardware is relatively simple — consisting largely of



Cray Research's C90: end of the line?

existing processors strung together by the hundreds or thousands — the hardware of traditional machines has become so complicated and temperamental that engineers who once improved performance by orders of magnitude overnight are now happy to achieve gains of a few per cent.

It is not for lack of effort. Both Seymour Cray and his protege Steve Chen, who left Cray in 1988 to form Supercomputer Systems Inc., have invested tens of millions of dollars into speedy gallium arsenide technology. But the technology has turned out to be more difficult than expected. Cray, for example, lost \$55 million last year struggling with the fragile chips, and neither company is expected to have its machines ready soon.

"I think Seymour and Steve Chen are barking up the wrong tree," says William Wulf, a University of Virginia computer scientist and former assistant NSF director for computer science. "There is no question about the demise of the behemoths."

Cray, Chen and the other traditionalists are up against nothing less than the laws of physics. Because the vector machines run one instruction after another, the only way to speed them up is to execute each instruction faster. With the speed of light and electric signals being an absolute, designers try to bring the parts as close together as possible and run them as fast as they will respond. The heart of the Cray 3,

for example, is no bigger than a shoebox and runs at more than 200 MHz. But warp speed and cramped components mean temperatures approaching the melting point for some parts. At least one vector machine has to run in a constant bath of liquid nitrogen lest it melt down.

In contrast, speeding up parallel machines is theoretically as simple as adding more cheap processors. Thinking Machines has made a computer with 64,000 processors, and even larger machines are available to the buyer with enough money.

Leading the endless quest for speed are what researchers call 'Grand Challenge' problems. These are attempts, such as climate prediction and DNA sequencing, to model reality on computers. "Because they come from nature," says Steger, "Grand Challenge problems have a high degree of parallelism in them." In other words, a lot of things happen at the same time in the real world, and keeping track of them takes a lot of processors.

But vector machines are still preferable for many of the problems for which researchers use supercomputers. Even for problems in which two-thirds of the operations could be conducted independently and simultaneously, traditional vector machine are orders of magnitude faster than parallel machines. Only when programs reach 98 or 99 per cent parallelism are massively parallel machines faster.

Few real-world programs reach that level. Most of the problems now being run with such impressive speed on massively parallel machines are what pundits call "embarrassingly parallel" — relatively unique simulations such as atmospheric modelling where the computer is required to recalculate conditions at thousands of unrelated data points. Although such programs are obviously important, the average program marches through its calculations one step after another — something massively parallel machines generally do no better than desktop PCs.

Because rewriting computer code to run on parallel machines is still a black art, experts predict that the traditional machines will last another decade. "The old guard isn't dead," says Bruce Steger, manager of the massively parallel programme at Cray Research. "Vector machines may be dinosaurs, but the dinosaurs lived for an awfully long time."

But even as it defends its old designs, Cray Research is working on a \$12.7-million grant from the Defense Advanced Research Projects Agency to hasten their demise. One goal is software that enables vector programs to run on parallel machines — just the thing to bury the traditional machines once and for all.

Christopher Anderson

The best machine for the money

Washington

REBOUNDED from their failure to obtain federal funding for a \$1,500 million burning plasma experiment (BPX), magnetic fusion scientists in the United States are closing ranks behind a machine that they think the government can afford.

The search for an alternative to the BPX may represent a new way to fund big science. Rather than coming up with an idea and trying to persuade politicians to support it, fusion scientists have worked furiously since last fall to find a project that will cost no more than the US Energy Department has decided it wants to spend at present on the long-range search for a technology that will generate more energy than it consumes.

The result is a machine that may be driven more by economics than by science. While it would maintain a US presence in magnetic fusion over the next decade, both scientifically and commercially, it falls far short of what many scientists would like to accomplish.

"Yes, this is a backwards approach to doing science," says Stephen Dean, president of Fusion Power Associates, which lobbies for the industry. "But we are living in an upside-down world, in which financial considerations take precedence over good science."

Next week, a task force will submit a report to an advisory committee on fusion energy that is expected to recommend that the government build a \$400-million Tokamak Plasma Experiment (TPX) at Princeton University. Princeton was to be the site for the cancelled BPX, and its scientists already operate the leading US tokamak.

The TPX, which has also been referred to as the steady-state advanced tokamak, would operate for up to thousands of seconds. That is a relatively long period of time compared to current efforts, which apply a brief pulse of enormous energy to the superheated plasma. It will use superconducting magnets to achieve the power-

ful fields needed to keep the plasma confined. Researchers hope the experiment will provide clues about the conditions in which ignition, a sustained fusion reaction that uses a mixture of deuterium and tritium, would be possible.

The BPX, on the other hand, was intended to reach ignition as a demonstration of the feasibility of a fusion nuclear reactor. Building such a reactor is the ultimate goal of a joint US, European, Russian, and Japanese effort called the International Thermonuclear Experimental Reactor (ITER). That reactor will be a \$6,000 million programme carried out in several stages and sites over the next 30 years.

Some scientists are still hoping for a machine, much more expensive than the TPX, that could address the technical aspects of achieving ignition as well as the underlying scientific questions. Such a machine, says Ron Parker of the Massachusetts Institute of Technology, would give the country more bang for its fusion buck.

"I see it as a choice between spending \$750 million for a machine that can generate energy and spending \$400 million for a machine that just does physics", he says. "And I'm afraid that, if they back TPX, that decision will close out any discussion of the subject for the next 10 to 15 years."

But most researchers believe that they do not have that option. They argue that the Energy Department would reject any proposal above the \$500-million limit recommended last fall by the Townes committee (named after its chairman, Charles Townes of Princeton). That recommendation was based on a projected real growth of 5 per cent in the annual budget for magnetic fusion.

"If Congress says that we can have a 10 per cent real increase, then I'd say, 'Let's look at Parker's machine'," says David Baldwin from Lawrence Livermore National Laboratory, who is chairman of the task force reporting to the Fusion Energy Advisory Committee. "But if we asked for a billion-dollar machine", which Baldwin

says may be too low for what Parker wants to build, "we'd be sent packing."

There is also an element of make-work in the proposed TPX. Existing experiments at Princeton will be winding down within a few years, says William Happer, head of the Office of Energy Research, and the research team there needs TPX if it is to remain intact. In addition, US companies need to hone their skills on TPX to remain in the running for the much larger contracts that will be awarded if ITER is ever built.

"Unless you have a national capability," says Happer, "you can't participate meaningfully in an international effort. We have to teach our industry to build superconducting magnets and vacuums, and the best way is to do it. That's what the Europeans are doing."

The government has requested \$20 million in 1993 to move ahead with plans for the TPX. Advocates say that at its peak TPX will cost the Energy Department roughly \$80 million annually, and that it could be running as early as 2000 if construction begins in 1994.

Many scientists are nevertheless worried that the TPX will, like its predecessors, again fall short of its goals. "You can't hide the fact that what you can do for \$400 million is a lot less than what you can do for \$1.5 billion", says Keith Thomassen, a fusion researcher at Livermore. And with each experiment more expensive than the last, there is fear that the federal government will one day decide that the necessary next step is too costly.

"We've been saying for a long time that fusion power is at least 30 years away", says Peter Politzer of General Atomics Corp. in San Diego, California, which operates the D-III-D tokamak machine. "And it would be a very dismaying prospect if we had to come back to Congress after finishing with the TPX and say that we need X billion dollars more, and that fusion is still 30 years away."

Jeffrey Mervis

Fusion on \$2 a day

For the price of a single US researcher, the US Department of Energy (DOE) will support for a year a team of 116 physicists and their fusion experiments. An agreement was worked out last month between DOE, the US fusion contractor General Atomics Corp. of San Diego, and the Russian Kurchatov Institute in Moscow.

The one-year, \$90,000 contract (about \$2 a day for each of the Russian scientists), will maintain a five-year collaboration between General Atomics and the researchers at the Kurchatov Institute. Since establishing working ties in the late 1980s, the researchers have visited each other often and now communicate regularly, says Michael Roberts, director of international programmes in the DoE office of fusion energy. Unlike its military cousin, inertial confinement fusion, magnetic fusion research has been unclassified since the 1950s.

The two teams are already working on complementary projects — both laboratories have tokamak fusion reactors and are concentrating on the problem of confining plasma with magnetic fields. Especially enticing to DOE was the fact that the Russian machine — known as T-10 — also has the world's most powerful plasma heater.

"What they have to offer is a working facility doing what we want done", says Roberts. "It's not a sustainable situation, obviously, but it's a good opportunity now."

Other groups at the Kurchatov institute are also seeking similar subsidies from the West (see *Nature* 351, 683; 1991). But this is thought to be the first time that the US government has agreed to the direct support of research in the former Soviet Union.

Christopher Anderson

Zero-sum game

BURTON Richter, the director of the Stanford Linear Accelerator Center (SLAC), has proposed halving his own budget to pay for a new accelerator that federal officials decided two months ago was too expensive to fund until later in the decade. But Richter, who believes that the new machine is essential to keep his laboratory at the forefront of research in the field, must first convince his colleagues in the high-energy physics community that the money is his to spend and that a \$150-million machine is the best way to use scarce federal funds.

Rather than wait for the US Department of Energy (DOE) to provide the money for a new collider to create subatomic B mesons, Richter hopes to take matters into his own hands. At a presentation last month to a priority-setting subcommittee of DOE's High Energy Physics Advisory Panel (HEPAP), Richter proposed to suspend the current SLAC experimental programme for six months of the year and use the \$70 million saved to build the new accelerator.

But Richter's view of the SLAC budget irritates many other physicists in the community. "He says it's his money — I say it's the biggest scandal in high-energy physics", says Thomas Kirk, head of the high energy physics division at the Argonne National Laboratory. "It's not God-given that every lab gets a certain budget", adds Charles Baltay, a Yale University physicist and a member of the team now running an experiment at SLAC.

In January, DOE and the National Science Foundation (NSF), the two agencies that have solicited proposals for what is being called a B factory, decided that the prospects for high-energy physics funding were too "bleak" to permit consideration of a B factory until late in the decade. Besides dashing SLAC's hopes, the decision put a halt to efforts to build a similar large machine at Cornell University, which has an NSF-funded accelerator facility. Cornell also plans to divert small amounts of its budget over several years to accomplish, in a less theatrical manner than Richter, the same end.

But SLAC, which has suffered an ignominious loss to the European Large Electron Positron detector in the race to create Z particles (see page 99), is facing more than just the loss of a new accelerator. With little prospect of doing new physics at the facility, many physicists are wondering whether even half its current budget is too much.

That question is in the hands of a subpanel of HEPAP, which met this week to examine priorities for the field. The subpanel will make its recommendations to the full advisory committee on 13 April.

Christopher Anderson

Tough times ahead

New Delhi

GOVERNMENT scientists in India will have less money to spend this year as a result of across-the-board cuts in the budget announced last week.

The 1992-93 budget, presented to Parliament in the midst of an economic crisis, provides for an increase for research and development that fails to keep pace with the country's 12 per cent annual rate of inflation. The new budget contains \$872 million for science, which represents about \$40 million less in purchasing power than the current budget. Those figures do not include \$390 million for defence research.

The budget squeeze has forced scientific agencies to re-define their priorities. The space department, which will receive \$190 million, has decided to spend \$25 million less this year on the development of satellites and on applied research. The atomic energy department now plans to build two nuclear power plants rather than six.

The Scientific Workers Association, representing the 8,000 employees in the 42 laboratories of the Council of Scientific and Industrial Research, has warned management that it will not tolerate any layoffs or laboratory closures as a way of helping to ease the budget deficit. Never-

theless, the deficit has already forced some scientists to look for new jobs. Last month, the government decided to close down the 34-year-old National Buildings Organization, which designs low-cost housing, sets building standards and conducts research on alternative materials. Some 110 scientists and engineers are losing their jobs, and have been encouraged to find work with nongovernment bodies.

The cuts, combined with a devaluation last October of the rupee, have also devastated laboratories that rely on materials from abroad. Many institutes have halved their subscriptions to foreign journals and their book purchases, as well as trimming their purchases of equipment and instrumentation.

There were signs of hard times for Indian science even before last week's announcement. In December, all departments were asked to abolish 10 per cent of their middle- and upper-level administrative posts; more than half of those posts are held by scientists and technology experts. The government has also refused to name a new head for the Department of Ocean Development in what may be a prelude to abolishing the department.

K.S. Jayaraman

GUPTA'S RETURN

Pressure grows on Panjab University

New Delhi

INDIA'S scientific community seems at last alerted to the implications of last month's surprise reinstatement of palaeontologist V.J. Gupta as a senior professor of the Panjab University. Meanwhile, at Chandigarh, the continuing investigation of Gupta's alleged misuse of fossil evidence has taken a bizarre turn: the few among Gupta's local colleagues who helped frame charges against him have been asked to explain why they are not equally at fault.

Three of Gupta's former colleagues are especially fearful of victimisation. Summoned to appear on 29 February before M.J. Gujral, a retired high-court judge who was appointed by the university to complete the investigation of the allegations against Gupta, they declined to appear. Arun Ahluwalia, one coworker-turned critic, says it is ironic that "those who have spoken the truth should have been put on the defensive" while Gupta is "firmly back in his chair".

The university explains that it reinstated Gupta before completing its investigation because it "can neither keep a confirmed employee on suspension indefinitely, nor act on allegations without observing the due process of law".

Those summoned before the Gujral

inquiry are drawn only from among Gupta's coworkers at the Panjab University, 14 of whom have stayed out of the controversy of the past three years. Ahluwalia believes that he and his fellow-critics are being held hostage for Gupta's reinstatement as head of department next month, and that the investigation will be biased by Gupta's 1,000-page defence of himself.

But the university's sleight-of-hand has excited scepticism elsewhere in India. A.S. Paintal was director of the all-India Institute of Medical Research whose expedition to the Himalayas last summer failed to confirm Gupta's claims. Speaking this week as president of the Society for Scientific Values, he said that "we will not tolerate a cover-up". If the university tried a "whitewash", he would go "right to the Prime Minister".

The University Grants Committee (UGC), the chief source of university funds, is also exercised. Its chairman, G. Rami Reddy, promises to make public the report drawn up by the expedition of the Geological Society of India, financed by UGC and three other agencies, "as soon as I can lay hands on it". Reddy says that UGC will cut off Chandigarh's funds if necessary.

K.S. Jayaraman

MRC follows NIH on patents

London

BRITAIN'S Medical Research Council (MRC) has decided to follow the US National Institutes of Health (NIH) in filing for patents to cover the complementary DNA (cDNA) fragments sequenced by its genome researchers. But the move, announced last week by Alan Howarth, UK science and higher education minister, is being taken reluctantly.

Britain would like the other countries with major cDNA sequencing efforts to agree by international treaty not to file for patents on genome sequences of unknown utility. But in the absence of such an agreement, MRC says it has no choice but to file for patents itself.

MRC officials last summer strongly criticised Craig Venter, a researcher at NIH, after he filed a patent covering more than 300 cDNA fragments. They argued that an attempt to claim patent rights for genes of unknown function, on the basis of incomplete sequences, ran counter to the spirit of international collaboration at the heart of the Human Genome Project (see *Nature* 353, 785; 1991). But the MRC has now taken the same route, now that Venter's original application has been followed by a second one, this time covering 2,375 genes (see *Nature* 355, 665; 1992).

The first MRC patent application will be filed within the next two weeks, and will cover more than 1,000 cDNA sequences (about half of the sequences identified by the MRC team). The main motivation is to ensure that Britain will not be at a competitive disadvantage if the NIH patents are granted.

MRC secretary Dai Rees says that the MRC intends to match the NIH move-for-move, filing its initial application in the United States, rather than with the European or British patent offices. This should mean that the NIH and MRC applications will stand or fall together. Dai George, from the Association of the British Pharmaceutical Industry, also believes that the US Patent Office is more likely than its European counterparts to grant patents for cDNA sequences. US patent examiners "take a broader view of what is patentable," he says.

British drug companies are reluctant to comment on the patent applications until they have studied their wording. Patent agents hired by the MRC are still working on the British application, and although Venter's original application has now been released by the NIH, British companies have not yet examined it.

The MRC application puts the council at odds with the Royal Society, which denounced cDNA patenting in a statement late last month. But Anne McLaren, the Royal Society's foreign secretary, says she accepts that the MRC had little choice

but to follow the NIH's lead if it hopes to protect the UK government's commercial interests.

The British move is expected also to focus attention on the other countries with major cDNA sequencing efforts. French research minister Hubert Curien has outlined his opposition to cDNA patenting in a recent letter to *Science* (254, 1710; 1991). A generalized system of patenting the human genome would be "ethically unacceptable", Curien wrote. "A patent should not be granted for something that is part of our universal heritage."

Japan, the other country with a sizeable cDNA sequencing effort, has yet to reveal its position. But if Japanese officials also choose the patenting route, the French research ministry would face formidable pressure to abandon the moral high ground. French companies are unlikely to be happy if forced to negotiate licences with foreign agencies to exploit cDNA sequence data, when their own government has been one of the most generous supporters of cDNA sequencing.

Nevertheless, Curien will be an important ally as the British government attempts to secure an international agreement to waive any cDNA patents that may be awarded. Howarth was expected to raise the issue this week, at a meeting in Paris of science ministers and advisers from the industrialized nations that make up the Organization for Economic Cooperation and Development. But with US officials showing strong support for the NIH patent applications, prospects for an agreement in the foreseeable future seem dim.

Whatever its long-term commercial implications, the MRC's decision to file for cDNA patents means that researchers should soon get access to the British cDNA sequence data. The MRC had intended to open its cDNA sequence database last November, but these plans were shelved after Venter's surprise patent application. Once a patent has been filed safely, however, the MRC will be able to release data for the sequences covered by the application without undermining its intellectual property rights.

Rees says that the MRC is reviewing its controversial scheme to charge commercial users a fee to subscribe to its cDNA database (see *Nature* 354, 96; 1991). He maintains that the plan was widely misunderstood, and was designed merely to get industry to contribute towards the cost of the database, rather than being an attempt to profit from genome research.

For their part, industry sources seem unconcerned. "We'd rather not pay," says one senior British pharmaceutical industry scientist. "But in this business, pragmatism rules."

Peter Aldhous

Instant initiative

EAGER to show his commitment to reviving a sluggish US economy as he campaigns for reelection, President George Bush last week told his science adviser, D. Allan Bromley, to begin a new research initiative on advanced manufacturing.

There have been five such initiatives since Bush took office in 1989. Each was announced as part of the president's annual budget request to Congress in January, and unveiled only after agency officials compiled a lengthy inventory of existing federal programmes and detailed plans of how the government should spend its money. In contrast, the manufacturing initiative was approved at a meeting on 2 March of an inter-agency coordinating council and announced three days later at a press conference, with no accompanying list of current projects and no description of research priorities. The White House has not even chosen a chairman for the panel that will oversee the initiative, according to Bromley.

J.D.M.

No SSC for India

THE new Indian budget (see page 97) contains no money to continue its support for the US Superconducting Super Collider (SSC). India has pledged \$50 million to the \$8,500 million proton-proton accelerator being built in Texas — the first and so far only foreign contribution — and research teams in India have been hard at work for nearly three years designing prototypes for various parts of the gigantic machine.

Roy Schwitters, director of the SSC laboratory, says he "knows nothing" of any change in policy, pointing out that the head of the Atomic Energy Department in India visited the laboratory a few weeks ago to discuss ways to expand collaborative efforts. Indian officials have declined to say whether last week's budget reflects a temporary omission or a fundamental change in policy.

J.D.M.

Activists burn lab

ANIMAL activists ransacked and burned the offices of a toxicology researcher at Michigan State University late last month, causing between \$50,000 and \$100,000 in physical damage and destroying years of research data. In the early-morning raid, the activists targeted both Richard Aulerich, a scientist investigating the effect of common environmental contaminants on mink, and a mink research farm, where the animals were housed. Aulerich's data and other files were opened and burned in the fire. People for the Ethical Treatment of Animals released a statement on behalf of the underground group Animal Liberation Front (ALF) that credited the raid to ALF members and described it as the first action by the group in Michigan.

C.A.

Another hole in ozone data?

Tokyo & Washington

ANOTHER gap in observations of the Earth's ozone layer may open up in the mid-1990s because of a decision by the Japanese government to delay the launch of an advanced Earth observation satellite.

Two weeks ago, Japan's Science and Technology Agency informed US and European partners that the launch of the Advanced Earth Observing Satellite (ADEOS) will be delayed one year, until early 1996. ADEOS will carry radio wave and optical sensors built by the US National Aeronautics and Space Administration (NASA) and the Centre Nationale d'Etudes Spatiales (CNES) of France, including one of NASA's Total Ozone Mapping Spectrometers (TOMS).

A decision to drop the planned launch of the German satellite ATMOS has already opened up a yawning gap in the mid-1990s in data on chemical changes in

the ozone layer (*Nature* 355, 662; 1992). The TOMS detector on ADEOS, which will measure the total amount of ozone in the column beneath the satellite, will be the fourth in a series. Because the missions have a projected life of only two years, NASA officials are concerned that the one-year delay could open up another gap in ozone observations.

The decision to delay ADEOS came after the space agency failed to get the full budget it was requesting for the satellite in fiscal year 1992, which starts next month. An agency official says the chief reason for delay is technical difficulties in getting the many sensors on the ADEOS platform to work together. But the Japanese have told NASA that the delay was for budgetary reasons and because of an agreement with the Japanese fishing industry.

David Swinbanks & Christopher Anderson

Solving a fishy problem

THE ability of fishermen to hold up the launch of Japan's rockets (see above) is just one example of their disproportionate political power in Japan.

When the National Space Development Agency (NASDA) established its launch site on the little island of Tanegashima off Kyushu, the southern main island of Japan, negotiations with local fishermen were a major hurdle. But with the help of a leading member of the ruling Liberal Democratic Party, an agreement was reached that provides the fishermen with ¥600 million (\$4.6 million) every year in 'compensation' for damage caused to their tuna fisheries by rocket launches. Of course, nobody knows whether the launches cause any damage to the fisheries.

In return, NASDA is allowed to launch during two, two-month periods each in summer and winter. Because the nearby space centre of the Institute of Space and Astronautical Science must fit into the same window, NASDA is effectively limited to two launches a year. The ability of fishermen to hold the government to ransom stems from the political power of fishing cooperatives. For example, before work began on construction of a man-made island off Osaka to accommodate a new 24-hour international airport, fishermen from nearby cooperatives were paid a total of over ¥40,000 million (\$307 million) before construction could proceed. **D.S.**

Z not

THE race to produce rare Z particles has turned into a blowout.

The big loser is the Stanford Linear Accelerator Center (SLAC), the US laboratory that had hoped to use a cheap and novel collider to beat its competitor, the Large Electron-Positron (LEP) collider at CERN, the European physics laboratory outside Geneva. It is now clear that such an approach has not worked.

Last month, at a meeting of an advisory panel to the US Department of Energy, SLAC reported that its new machine had produced only 1,600 "Znaughts" last year, of which just one-third were actually captured by a detector. During the same period LEP produced about a million of the particles. "It's no longer a race," concedes

William Kirk, SLAC's assistant director.

SLAC researchers were never able to transcend their new machine's tricky beam geometry and technical malfunctions, he says. Worse yet, the one advantage over LEP that SLAC seemed to have — a polarized beam — has not materialized. The polarized electron source that was installed a month ago did not work, and SLAC is now trying to design another. The replacement "better work out or it will be highly embarrassing," says Kirk.

That may be an understatement. With little on the long-term table but a controversial proposal for another new accelerator (see story page 97), SLAC is facing a future that contains virtually no world-class physics. **Christopher Anderson**

Small uncertainties

WELSH higher education has been thrown into confusion by the reorganization of funding arrangements for universities announced last year.

The surprise decisions that polytechnics would in future have the status of universities and that there would be a separate funding council for Wales as well as Scotland has provoked questions about future relationships between a handful of institutions. There is only one federal university and only one polytechnic (which hopes to become the University of Glamorgan).

One complication is that the polytechnic seems bent on resisting pressure to become the seventh constituent college of the federal university. Another is that some of the larger university colleges, notably Cardiff and Swansea (with 40 per cent and 22 per cent of the university's 20,000 students, respectively) hanker for separation.

In microcosm, Welsh uncertainties are those of British higher education at large. But they have been magnified by two developments: the decision that there should be a second funding council for Welsh further education, but with the same chief executive (Professor John Andrews of Aberystwyth); and the appointment last year of Professor Sir John Meurig Thomas as deputy pro-chancellor of the university, apparently with a brief to strengthen the federal structure.

The six colleges of the university (which include the Welsh National School of Medicine) at present deal separately with the Universities Funding Council (UFC) for the United Kingdom. Officials working on the new structure say that this direct relationship will continue, and that the new council will follow UK practice in the past few years by allocating funds on criteria linked with teaching (student numbers), research and graduate education. But the larger colleges fear the dilution of the research criterion.

The polytechnic, by contrast, behaves as if it can only benefit by remaining independent (having shaken off local authority control after the Education Act of 1986). Transmogrified from its status before the Second World War as a mining engineering college, it now has the equivalent of 6,000 full-time students, most following degree courses that often have a vocational slant.

The calculation seems to be that the polytechnic, with a smaller ratio of faculty members to students than most universities and with a substantial income from mostly commissioned research, cannot lose from the application of rules devised for universities. But inter-institutional politics may yet intervene, illustrating that British university planning now depends on second-guessing institutions not yet in existence.

John Maddox

Light at the end of the tunnel?

SIR — Although the facts in a recent article on the situation in the former Soviet Union (*Nature* 354, 339; 1991) are correct, I cannot agree that Russian science is close to death. Rather, I believe that the collapse of the Communist regime and its consequences suggest that the present situation in Russian science is not just a dark tunnel. Now there is the prospect of a light at the end of the tunnel.

The huge centralized militarily orientated bureaucratic machine that has been Soviet science for the past 70 years allowed no innovation. It allowed only members of the Communist party to make a career in science and valued the labour of a scientist as only a fraction of that of a blue-collar worker. This machine insulated national from international science and separated research (institutes of the Academy of Sciences) from education (universities). It has to be destroyed.

The destruction of the machine means not the death of Russian science, but rather its recovery. I do not believe that Russian scientists who were able to contribute significantly to human knowledge even under the Communist regime will be less able to make similar contributions in a system based on internationally accepted principles.

Another part of the story concerns the personal fate of Soviet scientists. While a large number may lose their jobs and will therefore have to find other social roles, it is also true that the present situation demands a drastic reduction in the number of unproductive scientific institutes as well as of the many unproductive scientists working in them. To increase efficiency, Soviet science must dismiss literally thousands of able-bodied people who have hitherto spent years in their institutes playing chess, drinking tea, solving crossword puzzles and participating in party and trade union activities, and whose expertise (assuming they possessed any) was lost many years ago.

Much the same will happen in other fields, but nobody expects that the discharge of thousands of people from the huge and ineffective *kolkhozes* (collective farms) will mean the death of Russian agriculture.

It is unlikely that really competent scientists will fail to find a place in a new system. No nation sacrifices education even in the hardest of times, so that universities in the future may become institutions at which more scientists will find themselves doing both research and teaching. It must also be hoped that the best research institutes will not be closed. The development of private business should also eventually lead to the

employment of a large number of researchers. And, last but not least, the new political changes allow young Russian scientists to train in leading foreign laboratories (as I am now).

The present difficult situation in Russia (including that in science) requires careful consideration as well as international goodwill. But the difficulties connected with the destruction of the old system are only one side of the coin. The other side is the hope for improvements stemming from the development of science in a free country.

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Estonia's plight

SIR — The sympathy that *Nature* demonstrates for ex-Soviet science deserves appreciation, but I am concerned that the ex-Soviet science that needs help always, in your pages, turns out to be Russian science. A recent letter from Moscow draws attention to the poor state of science "in our country (by which we mean the whole territory of the former Soviet Union, including the now independent republics)" (*Nature* 355, 384; 1992).

As an Estonian scientist I feel obliged to broaden the frame. I sincerely respect the best in Russian science, and it is a pity indeed that it is not compatible with the Russian economy. Nevertheless, for us, who have been living and working under Soviet Russian-speaking occupation, it is intolerable that our respected colleagues from Moscow still claim to speak on behalf of "the now independent republics". My home university in Tartu was founded by Swedes as the second Swedish university after Uppsala. As Universität zu Dorpat it was a well-known European centre of science run by the Baltic Germans, and after the First World War it became the Estonian national university. The politics of the Russian/Soviet Empire ruined it four times. (To be honest, it has provided help to rebuild it too, twice.) Since 1944, there have been a greater number of prominent ethnic Estonian scientists abroad than in Estonia. Nevertheless, Estonians formed less than 1/250 of the population of the Soviet Union, but 1/25 of the 100 most cited Soviet scientists.

The economic chaos left by socialism is even worse for the smaller cultures, where educational traditions have been

based on strong regional universities. The brain-drain is already devastating in the Baltics, because of the narrower culture gap with the West. We do not have huge well-equipped armies to feed, but we too are short of resources. But we have to hold out, since the fate of science in Estonia, Latvia and Lithuania (as elsewhere) determines, after all, the fate of these European nations.

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Homage to Tswett

SIR — M. S. Tswett is generally regarded as the sole discoverer of chromatography^{1,2}. But analytical and preparative frontal paper chromatography was reported in 1861 and published in 1862 by Friedrich Goppelsroeder³, professor of chemistry at Basel University. Goppelsroeder thought that an observation by Schoenbein on differential migration of pure compounds on paper dipped in a solution would give rise to a useful analytical tool he later called *Capillaranalyse* (ref. 4).

Is frontal paper chromatography really chromatography? It accords with the definition of chromatography by Gordon *et al.*⁵ as a "technical procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material, irrespective of the nature of the physicochemical processes that may lead to the separation of substances in the apparatus". The most remarkable features of Goppelsroeder's work are repeated analysis, allowing "sharp separation of mixtures of dozens of compounds", identified by spectral data (*sic*); use of impregnated papers and other media; extensive use of specific reagents and fluorescence; spectral examination *in situ*; and analysis of organic and inorganic compounds, including proteins.

I believe that Tswett's important contribution was the development of initial narrow-zone and column chromatography, which allowed the development of modern techniques. Goppelsroeder did not merely perform some curious experiments on filter paper: he had a deep insight into the possibilities of the method he discovered.

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1. Ette, L. S. *Int. Lab.* 21(8), 18–24 (1991).
2. Chovin, P. in *Chromatographie en Chimie Organique et Biologique* (ed. Lederer, E.) 1, 1 (Masson, Paris, 1959).
3. Goppelsroeder, F. *Bull. Soc. Ind. Mulhouse* 32, 116–121 (1862).
4. Goppelsroeder, F. *Studien über die Anwendung der Capillaranalyse* (Birkhäuser, Basel, 1904).
5. Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. *Biochem. J.* 38, 65–68 (1944).

Ethical scores for animal experiments

David G. Porter

Scientists have for too long been faced with a polarized debate concerning the ethics of animal experiments. An ethical ideal and a practical scoring system would be valuable for the moderate majority.

MANY scientists probably occupy the middle ground between the absolutists at either extreme of the animal-rights debate. Neither the abolitionist nor the 'no-restriction' advocate offers a solution to the ethical problem of the use of animals in science that is both practicable and humane. Moderates are left in an ill-defined ethical no-man's-land in which a great deal of philosophical analysis is available, but little in the way of practical advice.

Those who experiment on sentient animals face the dilemma that the process of investigating the very 'material' that they wish to study carries with it an ethical dimension. This is hardly the case for the physicist or chemist, or even the botanist. An ethical content has long been recognized to be part of research on human beings, and most scientists feel repugnance at human experimentation without ethical restraint. But there is no coherent philosophy for dealing with animals, and scientists have generally subscribed to a largely unarticulated dogma that 'humans come first' and that biomedical science is essential for human well-being, if not survival¹. Animals have been seen primarily as tools for research, a practice that is defended almost exclusively by a litany of 'good things' that emanates from it. Contrary to allegations made by some critics of modern medical research², 'good' things have come from animal experimentation, and, although their significance might be exaggerated by researchers, even in good faith, they have been fundamental to the understanding of biological mechanisms and to improvements in medical care. But, a list of 'good things' is not always an effective counter-argument to a philosophical challenge. The defence of slavery in the eighteenth and nineteenth centuries by extolling the 'good' things about the institution, illustrates this point. Indeed, scientists' defence of animal experiments is surprisingly simplistic for a section of society that prides itself on its intellect.

Ethical approach

We can develop ethical tools to deal with the problem of whether or when to use animals in experiments only if we first have an ideal. An ideal of the animal-rights movement, which might better be referred to as the 'Schweitzerian' ideal, as it is close to that advanced by Albert

Schweitzer, is that we should avoid harming sentient animals whenever possible. The scientific community and government³ tend to regard this as unattainable and usually conclude that it is not worth serious consideration. All ideals are probably unattainable, but their value lies in providing yardsticks by which to judge our conduct. I believe scientists should adopt the Schweitzerian ideal of respect for all life, and become anti-vivisectionists at heart. Thereby, we will deliberately create a tension between ideal and practice similar to that governing our behaviour in many other activities. Some might find this outrageous, but the onus is on them to explain why we would not want to share an ideal that seeks always to minimize or avoid the harm we inflict on sentient animals.

Once we adopt this philosophy we need a 'tool-kit' to help apply the philosophical ideal to the practical environment of the laboratory. I propose a simple scoring system to explore the experiment from the viewpoint of the research animal. Like any scoring system it suffers from limitations and has the disadvantage of implying accurate measurement where there can be none. It should, nonetheless, draw the attention of the experimenter to a range of factors that affect the animal beyond those usually considered. The experimenter is encouraged to imagine, as objectively as possible, what it is like to be a rat, a dog or a monkey about to experience the experiment. The tool-kit's novelty is that it advocates a change in philosophy and then challenges the investigator to ensure that the proposed experiments are of a quality and exigency sufficient to justify the total demand that will be made of the animal. A similar holistic approach, but lacking a scoring system, has been advocated recently for animal-use review committees⁴.

The premise of my scoring system is that every experiment on a sentient animal represents a departure, however small, from the Schweitzerian ideal. The more points scored, the further the departure. The ideal should generate constant pressure to avoid experiments on animals wherever possible, to seek alternatives, and to reduce the cut-off for unacceptable experiments.

Six categories deal with factors likely

to cause the animal pain or distress, and two assess the exigency and quality of the experiment. One of the animal categories (C) deals with the species to be used. A scale of 1 to 5 ranges from apparently relatively insensitive creatures to highly sensitive and intelligent mammals. The inclusion of animals as 'lowly' as molluscs draws attention to the difficulty of drawing a moral distinction between different life forms. Perhaps a unit of ethical concern (an ethicon?) is needed where a mollusc might score one and a chimpanzee 1,000,000. The scale could thus be modified, and should be revised as understanding is gained of the sentience of different living creatures, a principle embodied in existing UK legislation⁵. The objective of C is to demonstrate the extent to which we depart from our ideal when we harm a chimpanzee, while reminding us of a finite, if small, departure when we harm a mollusc.

Pain

Category D considers pain. Despite recent interest in assessing animal pain⁵, this is still difficult to gauge with certainty. The LASA working party report⁶ provides a severity index which could be used to score procedures in category D. Wherever there is doubt, the ideal should impel us to err in favour of the animal. For example, would investigators who score an intraperitoneal injection as 1 or 2 for a rat accept that score for themselves? Is hypophysectomy, a routine operation in many laboratories, in the low-pain category and is analgesia administered for it? Is the experimenter skilled in the planned procedure so that inadvertent suffering is avoided?

Category E refers to factors that may involve distress, discomfort or anxiety. To what extent will the animal be restrained, deprived of social contact, or placed in an unfamiliar, frightening, environment? How will its normal behaviour patterns of feeding, sleeping and exercise be disturbed?

Duration, in Category F, relates the length of the experiment to the animal's normal lifespan. A month-long experiment places a much greater demand in terms of lifespan on a rat than on a dog or a sheep. Therefore, we need to factor the animal's lifespan into estimates of the overall effect of an experiment. Category F assumes that animals 'per-

PROPOSED SCORING SYSTEM TO MINIMIZE SUFFERING IN ANIMAL EXPERIMENTS

A Aim of experiment

1. Alleviation of substantial human or non-human pain
2. Alleviation of moderate human or non-human pain or suffering
3. Clear benefit to human or non-human health or welfare.
4. Some benefit to human or non-human health or welfare.
5. Fundamental research for the advancement of knowledge (no clear alleviation of pain or benefit to human or animal health)

B Realistic potential of experiment to achieve objective

1. Excellent
2. Very good
3. Average/fair
4. Limited
5. Very limited or impossible to assess

C Species of animal

1. Low sensibility/consciousness
2. Some sensibility
3. Sentient but possibly limited consciousness
4. Sentient and conscious
5. Sentient, highly intelligent and precognitive

D Pain likely to be involved

1. None
2. Minimal/slight
3. Moderate
4. Considerable
5. Severe

E Duration of discomfort or distress

1. None or very short
2. Short
3. Moderate
4. Long
5. Very long

F Duration of experiment

1. Extremely short 10^{-5} LS
2. Short 2×10^{-4} LS
3. Moderate 2×10^{-2} LS
4. Long 2×10^{-2} LS
5. Very long $> 2 \times 10^{-1}$ LS

G Number of animals

1. 1–5
2. 5–10
3. 10–20
4. 20–100
5. > 100

H Quality of animal care

1. Excellent
2. Very good
3. Average
4. Good
5. Poor

C. Examples of animals are as follows: group 1, molluscs; group 2, cephalopods, fish, amphibia; group 3, reptiles; group 4, mammals (except group 5), birds; group 5: primates, carnivores, cetaceans. (Primates are not all as sentient as the anthropoid apes, but most are endangered species. Unless an additional category is developed to reflect the survival prospects of species, all primates are probably best left in group 5.)

D. Laws in many countries require that pain be of short duration. Assessment of scores under D should take into consideration the use of analgesics, together with current opinion of their effectiveness. It should also estimate factors such as post-operative pain, and the skill of the experimenter in the proposed procedures.

E. Considers all aspects of the procedure, for example, restraint, deprivation of social contact, unfamiliar environments, exposure to thermal stress, and so on.

F. LS, estimated normal lifespan.

H. Evaluates all aspects of the animal's environment while not under experiment. It includes, for example, quality of environment and its appropriateness for specific animal(s), caging, skill of attendants, quality of post-operative care, lighting, ventilation.

ceive' time in relation to their natural lifespan, an assumption for which we have no information, but is made on the 'benefit of doubt' principle.

Whether death *per se* is an 'evil' is debatable, but to kill 100 animals in an experiment represents a greater departure from the ideal than to kill five (category G). It is not always easy to score category G appropriately, because using more animals for a shorter period (category F) could generate lower scores than fewer animals used for longer. This is a dilemma that no scoring system can resolve, for there is no ethically acceptable number. Experiments should be statistically designed so that excessive numbers of animals are not used and that unnecessary repetition does not occur — see, for example, ref. 7.

The standard of care that surrounds the animal throughout its stay in a research establishment should be scored (category H). With greater emphasis on animal-care committees and sophisticated animal facilities, individual researchers may overlook this facet of an animal's experience. Modern animal facilities do not always provide the best environment for the experimental animal. How many rabbits exist in isolation confined to small cages and how many intelligent, inquisitive and highly social

primates live out impoverished lives deprived of contact with their peers and of environmental stimulation?

Scoring the science

Category A challenges the morality of inflicting pain unless the objective of an experiment is specifically to bring about the reduction of pain or suffering. The ideal steers us away from causing pain in the pursuit of profit (for example, enhancing animal production) or curiosity (see below) or to reduce minor discomforts of human beings.

Category A recognizes the need for 'curiosity-driven' or fundamental research, not directed at medical or health problems. Such research has often led to unexpected, valuable discoveries, but given the fairly high probability that it will not do so, it is difficult to quantify the inflicting of suffering in such inquiry. Its weighting therefore requires experiments to have low costs in the remaining categories, in keeping with the ideal.

Finally, category B scores the quality of the science, and requires an estimate of its chances of achieving its goal — that is, how far it is removed from its ultimate objective. It demands that the experiment be well planned and statistically sound and seeks a realistic judgment of its exigency. Experiments very

close to a pain-reducing objective would score lower than those at an earlier stage in the often tortuous investigative path.

The final score

The next step is to arrive at a total score out of a maximum of 40. The minimum unavoidable score of eight reflects the tension between ideal and practice and is a reminder that every experiment on a sentient animal represents a departure, in at least some measure, from the ideal.

The cut-off score should be as low as possible. Because the animal categories account for up to 30 points their contribution should be no worse than 15. Categories A and B should contribute no more than 7 together, giving a working cut-off of about 21. If experiments are carried out *in vitro*, without previous procedures on the animal *in vivo*, only categories A, B, C, G and H would be scored and a cut-off point of around 16 could be used. Under the imperative of the ideal, the cut-off score should be revised downwards as time goes on. Additional categories could be made, for example for endangered species.

By adopting the compassionate 'animal rights' ideal to guide their experimental studies, scientists would strengthen their ethical position. The proposed tool kit would eliminate some experiments that might be acceptable under the current law, and a finite loss of knowledge could result. Although it is important not to stifle our quest for new knowledge, ethical standards should be enhanced by developing new ways to advance knowledge with less animal suffering. Any setback resulting from ethical restraint would probably be transitory. Scientists are ingenious and industrious and would redirect their activities towards experiments with lower scores, or to the use of different experimental approaches, or even to the study of problems in human and animal health that are under-investigated but do not demand invasive experiments. □

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1. McLaughlin, R.M. in *Animal Research, Animal Rights, Animal Legislation* (ed. Concannon, P. W.) 11–15 (Society for the Study of Reproduction, Champaign, 1990).
2. Sharpe, R. *The Cruel Deception: The use of animals in medical research* (Thorsons, Wellingborough, Northamptonshire, 1989).
3. Scientific Procedures on Living Animals (Her Majesty's Stationery Office, London, 1983).
4. Prentice, E. D., Crouse, D. A. & Rings, R. W. *Invest. Radiol.* **25**, 271–274 (1990).
5. Morton, D. B. & Griffiths, P. H. M. *Vet. Rec.* **116**, 431–436 (1985).
6. LASA Working Party Report *Lab. Animals* **24**, 97–130 (1990).
7. Mann, M. D., Crouse, D. A. & Prentice E. D. *Lab. Animal Sci.* **41**, 6–14 (1991).

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Alzheimer's disease and amyloid

Two well publicized papers reporting transgenic mouse models for Alzheimer's disease have been retracted. But this setback may not be quite so serious as press reports have suggested.

THE amyloid precursor protein (APP) is a transmembrane glycoprotein of unknown function that is produced in most tissues, including the neurons and blood vessels of the central nervous system. An insoluble C-terminal degradation product, beta amyloid, accumulates in the ageing brains of humans and some other mammals and forms extracellular plaques. Dense amyloid plaques in aged brains may sometimes be associated with intracellular fibrils of different composition known as neurofibrillary tangles, accompanied by some neural degeneration. In Alzheimer's disease, which affects about 10 per cent of people in their seventies and 30 per cent in their eighties, very pronounced plaques, tangles and neuronal degeneration are accompanied by dementia. These observations have led to the hypothesis that the accumulation of beta amyloid itself is responsible for the progressive neuropathology and ultimately loss of cognitive function.

If this is the case, then a transgenic mouse expressing an extra amyloid gene in its brain might develop the same pathology, thus both establishing the role of amyloid deposits in the aetiology of the disease and providing a model on which to test potential therapeutic drugs. Three such transgenic animals have been reported, two of them showing what appear to be amyloid deposits^{1,2} and the third, published by Kawabata *et al.* in *Nature*³ apparently also showing intracellular neurofibrillary tangles and neuronal degeneration. Kawabata *et al.* have now retracted their paper on the grounds that they cannot repeat the neuropathological observations⁴, originally made by Higgins, who is the subject of an NIH investigation⁵; Wirak *et al.*¹ will also shortly retract their paper because it has turned out that the abnormalities they saw are characteristic of the strain of mouse they used; the amyloid staining was probably an artefact. The remaining paper, by Quon *et al.*², still stands; but although their transgenic mice show amyloid deposits in cortex and hippocampus, they show no other pathology and so at this stage neither establish the role of amyloid in Alzheimer's aetiology nor provide a model for testing drugs.

The two retractions have inevitably raised questions about the conduct of research and publication practices. The best time to take up these questions is

when the NIH enquiry on Kawabata *et al.* is complete, although it is important that in both cases the problem was brought to light by the vigilance of others in the field and that retraction followed promptly. In the meantime perhaps more important is the question of how this dual disappointment affects the understanding of Alzheimer's neuropathology and the hope of progress.

There is in fact remarkably little understanding of the mechanism of Alzheimer's neuropathology, leaving room for several different theories about the role of amyloid in its aetiology. It is possible for example that amyloid deposits in the blood vessels rather than in the neurons do the damage; and although there is some evidence that beta amyloid is directly toxic to neurons *in vitro*⁶, there is controversy over its effects *in vivo* and some believe the release and accumulation of beta amyloid is the consequence and not the cause of neural death. The transgenic mice of Quon *et al.* were designed to test yet another hypothesis — that Alzheimer's disease might result from an abnormal balance of the three normal isoforms of APP. They therefore used a transgene encoding one isoform, beta APP751, high relative levels of which have been reported by some in Alzheimer's disease. This is distinct from the other principal isoform, beta APP695, in containing an N-terminal serine protease inhibitor domain. The function of this domain and its contribution, if any, to the neuropathology of Alzheimer's disease, again, are unknown.

The special significance of the Kawabata *et al.* transgenic mouse was that it seemed to show characteristic Alzheimer's pathology and thus to be the best model of the three. Yet even had it held up (the suggestion is that the slide showing the neuropathology was in fact from human Alzheimer's brain), its credentials as a model were not perfect. As Dennis Selkoe pointed out in an editorial accompanying the publication of the paper in *Nature*⁷, the transgene they used encodes a C-terminal amyloid fragment that is never made physiologically. The mouse was therefore at best imperfectly mimicking the process that produces amyloid plaques in man. Selkoe also points out that, unlike normal APP genes, their transgene was under the control of a promoter that allows its expression only in neurons, at levels that

might be toxic for reasons other than those operating in Alzheimer's. It is unclear however whether the neurones are affected by the excess amyloid at all — always assuming that the protein itself is actually made: Kawabata *et al.* have shown expression only at the RNA level.

The best evidence implicating the amyloid protein in neuropathology probably now lies with recent studies in human genetics. In a small minority⁸ of families with an hereditary predisposition to Alzheimer's disease, a missense mutation close to the C-terminal end of beta amyloid segregates with the disease phenotype⁹⁻¹². Alzheimer's disease in these families follows an autosomal dominant pattern, which means only one mutant copy of the gene should be needed to produce the abnormality and a mouse model could thus be made by the introduction of a mutant transgene without the need to eliminate the normal gene.

Although such a mouse might provide a better test of the role of amyloid than one carrying an extra copy of a normal gene, as in the Quon *et al.* mouse, dosage effects are in fact also implicated by human genetics. Trisomy 21 in Down's syndrome confers an extra copy of the normal APP gene and presumably as a consequence, amyloid plaques accumulate, with full Alzheimer's neuropathology by the 5th decade. It may be that an animal with a lifespan as short as a mouse's can never develop a pathology that in man takes decades.

In the meantime almost everything remains to be learned about the processing of APP at the cellular level and its function at any level; and in the circumstances, the collapse of a possible partial Alzheimer's model may seem to leave us little further back than we were.

Miranda Robertson

- Wirak, D. O. *et al.* *Science* **253**, 323-325 (1991).
- Quon, D. *et al.* *Nature* **352**, 239-241 (1991).
- Kawabata, S., Higgins, G. A. & Gordon, J. W. *Nature* **354**, 476-478 (1991).
- Kawabata, S., Higgins, G. A. & Gordon, J. W. *Nature* **356**, 23 (1992).
- Marx, J. *Science* **255**, 1200-1202 (1992).
- Yankner, B. A., Duffy, L. K., & Kirschner, D. A. *Science* **250**, 279-282 (1990).
- Selkoe, D. *Nature* **354**, 432-433 (1991).
- van Duijn, C. M. *et al.* *Lancet* **337**, 978 (1991).
- Goate, A. *et al.* *Nature* **349**, 704-706 (1991).
- Naruse, S. *et al.* *Lancet* **337**, 978-979 (1991).
- Chartier-Harlin, M. C. *et al.* *Nature* **353**, 844-846 (1991).
- Murrell, J., Farlow, M., Ghetti, B. & Benson, M.D. *Science* **254**, 97-99 (1991).

Ultraviolet on the increase

Paul J. Crutzen

It is almost a truism: a loss of stratospheric ozone means there will be more harmful ultraviolet radiation (UV-B) at the Earth's surface, more skin cancer, a loss in crop yields and additional biological hazards. During the past few years, however, a number of articles have shown that the issue is not so simple: on regional scales, other factors such as tropospheric ozone¹, sulphate particles² in the atmosphere and even cloudiness can produce the opposite effect. Now Madronich of the US National Center for Atmospheric Research reports³ the first estimates of the intensification of UV-B radiation over the globe due to the measured losses of ozone in the years 1979–89.

The method adopted by Madronich is purely theoretical. A direct observational approach is not yet possible because, apart from the Robertson–Berger meters which are heavily weighted towards longer wavelengths, there is no integrated global network of monitors for ultraviolet radiation. Madronich's calculations are based on the total ozone trends for 1979–90 reported last year by Stolarski *et al.*⁴ (Fig. 1). These are derived from data from the satellite-borne Total Ozone Mapping System (TOMS) which integrates the ozone concentrations over the depth of the atmosphere. The greatest relative ozone losses have occurred over the Antarctic, approaching an average annual depletion of 3 per cent for the months of September to November. Indeed this peak depletion is so severe that it reverses the natural ozone cycle in which high latitudes should have the highest ozone levels in late-winter and spring; now spring is the time of minimum ozone in the Southern Hemisphere. In the Northern Hemisphere, the natural cycle is still followed, with the ozone minimum occurring in September

and October. But again, the CFC-dependent depletion is strongest north of 40° N, peaking at 1 per cent a year from January to April.

These data suggest that the biosphere has remained quite well protected from the damaging effects of solar ultraviolet in the northern spring, despite the strong depletion, and this is borne out by Madronich's calculations. Madronich divides the solar spectrum in the 280–400-nm wavelength region into 1-nm intervals and calculates the flux transmitted through the atmosphere for aerosol- and cloud-free conditions, so considering only absorption of ultraviolet by ozone and molecular (Rayleigh) scattering of radiation. (He has shown previously that increases in ultraviolet flux attributable to ozone loss are practically independent of environmental conditions such as cloud and aerosol distribution, so long as these factors remain constant.) As the TOMS data of Stolarski *et al.* give only the total column density of ozone over any particular point, Madronich has to assume a vertical distribution for his calculations: he chooses the US 'standard atmosphere' formula, suitable for mid-latitudes, and scales it to the total ozone measured at each latitude.

The final step is to weight the transmitted flux according to the biological damage it can do, which is greatest at wavelengths of 280–320 nm. As Fig. 2a shows, the largest absolute increases in DNA-damaging ultraviolet have occurred in southern-polar regions during September and November. However, substantial increases have also occurred in the Northern Hemisphere as far south as 30° N during February and March. Actual measurements⁵ at Palmer Station at 64.8° S in the Antarctic during the spring months indeed show marked increases in the ratios of the irradiances at wavelengths shorter than 325 nm relative to those at 350 nm, with larger values towards shorter wavelengths, in agreement with the calculations, confirming the theoretical approach of Madronich.

To put these results in perspective, Fig. 2b shows typical daily DNA-damage doses applicable for the year 1979. The maximum increase of the dose in the Antarctic spring is clearly only 7 per cent of the total typically received at the Equator. If life everywhere has established the same relative sensitivity to ultraviolet radiation, then the impact of the ozone depletions would be rather moderate. But there is no good reason for such optimism. Just as white-skinned humans are more prone to skin cancer than black people living in the tropics,

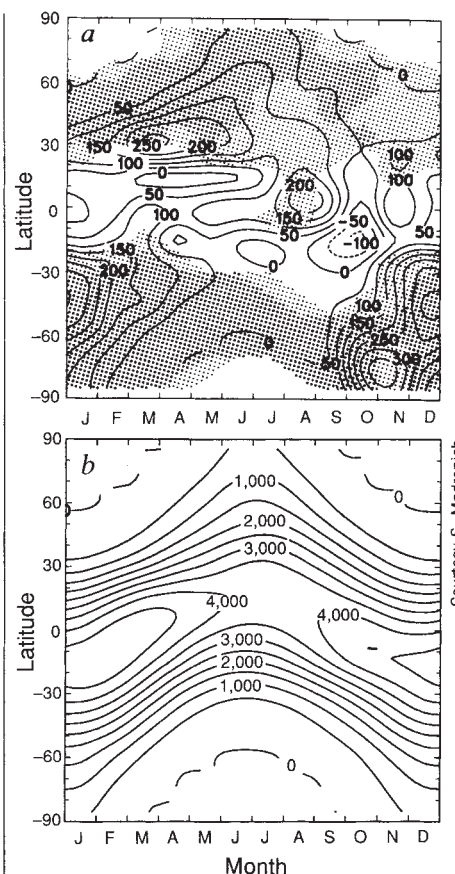


FIG. 2a Trends in daily effective ultraviolet dose for DNA damage as deduced by Madronich³ ($\text{J m}^{-2} \text{ day}^{-1}$). Dark and light shading, regions where the trends differ from zero by more than two and one standard deviations, respectively. b, Typical daily DNA-damage doses for the year 1979.

one may expect the biosphere at mid- to high latitudes to be more sensitive to the effects of losses in protective ozone. It is thus an important (but difficult) task to obtain reliable information on UV-B damage for various plant and animal communities and ecosystems. Although such research is under way, results applicable to real-life conditions are still rather inconclusive with the exception of the effect on human skin cancer. Results just published⁶ on phytoplankton productivity inside and outside the ozone-hole region indicate a reduction by at least 6–12 per cent due to the ozone losses during the spring months in 1990.

Of course, the stratospheric ozone levels considered by Madronich are not the only factor influencing the pressures of UV-B on the biosphere. Increased loading of particulate sulphate would counteract Madronich's trend, but measures to regulate SO_2 emissions, introduced in the 1980s, have halted this change. And ozone in the troposphere, an industrial pollutant, is (molecule for molecule) a stronger absorber of ultraviolet than ozone in the stratosphere. But even this has not increased so much as to nullify the stratospheric depletion. In any case, these mechanisms would

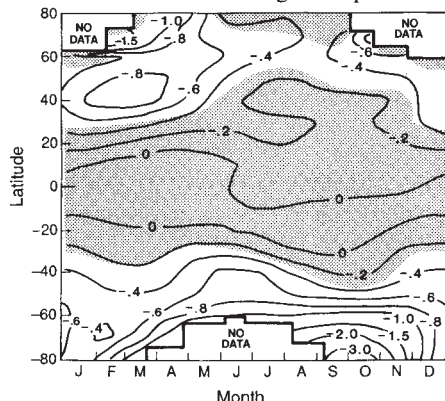


FIG. 1 The mean percentage depletion in total ozone over the latitudes shown for the months shown for 1979–90, as recorded by Stolarski *et al.*⁴ using TOMS data.

operate only at a regional scale, whereas the stratospheric effects are global.

All this equivocation arises because of our current failure to measure the changes in UV-B irradiation directly. What is needed is a worldwide network of UV-B monitoring stations with suitable spectral resolution to complement the ozone-monitoring Dobson network, as is being discussed at a meeting this week*. Indeed, as raised ultraviolet

fluxes affect atmospheric chemistry⁷ (by generating hydroxyl radicals which remove many atmospheric trace gases, such as industrial methylchloroform⁸), such a network would have scientific as well as health applications. □

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* UV-B Monitoring, Washington DC, 10–12 March 1992.

1. Brühl, C. & Crutzen, P. J. *Geophys. Res. Lett.* **16**, 703–706 (1989).
2. Liu, S. C., McKeen, S. A. & Madronich, S. *Geophys. Res. Lett.* **18**, 2265–2268 (1991).
3. Madronich, S. *Geophys. Res. Lett.* **19**, 37–40 (1992).
4. Stolarski, R. S., Bloomfield, R. D., McPeters, R. D. &

- Harman, J. *Geophys. Res. Lett.* **18**, 1015–1018 (1991).
5. Frederick, J. E. & Alberts, A. D. *Geophys. Res. Lett.* **18**, 1869–1872 (1991).
6. Smith, R. C. *et al.* *Science* **255**, 952–959 (1992).
7. Thompson, A. M. *Nature* **352**, 282–283 (1991).
8. Prinn, R. G. *J. geophys. Res.* (in the press).

DNA STRUCTURE

The quadruplex and the vase

Maxim Frank-Kamenetskii

SINCE Jane Richardson first proposed the 'vase ornament' classification of protein-folding patterns¹, the notion of the 'Greek key' has become common in the field of protein structure. The first X-ray data on the quadruplex folding of the *Oxytricha* telomeric sequence, presented by Kang *et al.* on page 126 of this issue², indicate that the same notion may

their mode of synthesis and their structure. The very ends of telomeres carry single-stranded DNA tails. DNA quadruplexes are attracting increasing attention because they readily form from single-stranded DNA molecules with telomeric sequences (general motif (T/A)_mG_n, where *m* is 1–4 and *n* is 1–8). In the quadruplex, four guanines cohere through Hoogsteen pairing. Until very recently, evidence of quadruplex formation was based on the data from gel mobility or from chemical probing^{3,4} (see also my previous News and Views article on the subject⁶). Although these results left few doubts, if any, that the quadruplexes were actually formed, they could not be used to elucidate the detailed geometry. All conjectures as to geometry (reviewed in ref. 7) were based on model building, symmetry considerations and conventional wisdom.

The proposed structure which emerged from this work corresponded to the Greek-key topology. In it, all adjacent strands in the quadruplex were thought to be antiparallel. The nucleotide conformation alternated in the *syn-anti-syn-anti* manner within the G-quartet, and was assumed to be the same along the strands.

The X-ray data now obtained² for the intermolecular quadruplex formed by two *Oxytricha* telomeric sequences G₄T₄G₄ confirm most of these features and provide us with a detailed view of the structure. The stacked guanine tetrads form a right-handed helix which, if extended, would give about 13 tetrads for each turn. The *syn* and *anti* con-

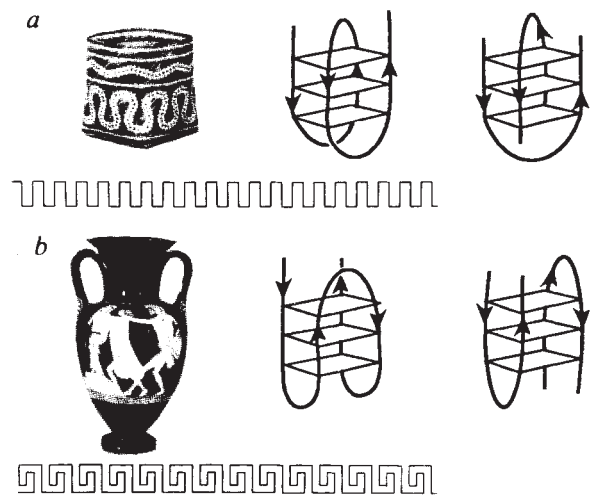
formations indeed alternate within tetrads, but in contrast to the early model the *syn-anti* alternation occurs along the strands. Such alternations follow also from the NMR data^{5,8}, though the reason for them remains obscure. Again, as expected, the potassium ion was found to be situated axially within the channel formed by the stacked tetrads².

Surprisingly, however, in their NMR study Smith and Feigon⁵ arrive at quite a different conclusion. Instead of the Greek key, they claim to identify Indian-key topology because the Overhauser interaction between the H8 atoms of guanines they observe is inconsistent with antiparallel orientation of all adjacent strands. In the case of the Indian key, the adjacent strands are both parallel and antiparallel (see figure). The nucleotide conformation in the G-quartet is *anti-anti-syn-syn*, not *anti-syn-anti-syn*, in the case of the Indian key.

Why should the X-ray and NMR data point to such different conclusions? The X-ray data are solid, and the arguments based on the NMR results, though much less direct, also look pretty convincing. So the most likely and most interesting possibility is that both groups are right. That would mean that quadruplexes are able to adopt a different topology and geometry under different ambient conditions. The most obvious difference between the two sets of experiments is that Kang *et al.* used potassium, magnesium and spermine as the counter-ions, whereas Smith and Feigon used only sodium. Support for the notion that different quadruplex structures may be adopted by the same telomeric sequences in the presence of different ions follows from the spectroscopic and photochemical data from Cech's group^{3,9}.

The new findings emphasize how quickly unveiling of the variety of DNA structures is showing that they approach protein structures in versatility — another example is the 'eclectic' structure proposed by myself and colleagues, in which a highly unusual DNA triplex and quadruplex coexist¹⁰. □

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a, Indian and, b, Greek vase ornamentation, with examples of the corresponding intra- and intermolecular folding of telomeric sequences forming quadruplexes.

become equally popular among those interested in unusual DNA structures. In accordance with the previously accepted model of the quadruplex structure in telomeres^{3,4}, the *Oxytricha* telomeric sequence folds in the Greek-key manner (see figure) with a potassium ion situated axially within the channel formed by the G-quartets. There is a 'but', however, for NMR data, reported by Smith and Feigon also in this issue⁵ (page 164), show that in the presence of sodium ions the *Oxytricha* telomeric sequence adopts a different, 'Indian key' topology.

Telomeres are the ends of chromosomes, and are highly unusual in both

1. Richardson, J. S. *Nature* **268**, 495–500 (1977).
2. Kang, C.H., Zhang, X., Ratliff, R., Moyzis, R. & Rich, A. *Nature* **356**, 126–131 (1992).
3. Williamson, J. R., Raghuraman, M. K. & Cech, T. R. *Cell* **59**, 871–880 (1989).
4. Sundquist, W. I. & Klug, A. *Nature* **342**, 825–829 (1989).
5. Smith, F. W. & Feigon, J. *Nature* **356**, 164–168 (1992).
6. Frank-Kamenetskii, M. *Nature* **342**, 737 (1989).
7. Guschlbauer, W., Chantot, J.-F. & Thiele, D. *J. Biomol. Struct. Dyn.* **8**, 491–511 (1990).
8. Wang, Y. *et al.* *Nucleic Acids Res.* **19**, 4619–4622 (1991).
9. Zahler, A. M., Williamson, J. R., Cech, T. R. & Prescott, D. M. *Nature* **350**, 718–720 (1991).
10. Voloshin, O. N. *et al.* *J. Biomol. Struct. Dyn.* **9**, 643–652 (1992).

An ape from the south

Peter Andrews

DISCOVERIES of fossil hominoids from the first half of the Miocene, 24 to 15 million years ago (Myr), have been restricted mainly to eastern Africa. On page 144 of this issue¹, Conroy *et al.* describe a fossil hominoid jaw from the Miocene of Namibia, thus extending the range of Miocene hominoids down well into southern Africa. The specimen will be the centrepiece of a workshop, *Apes or ancestors?*, to be held at the American Museum of Natural History, New York, on 28 March.

Combined with other discoveries in northern Kenya^{2,3} and Saudi Arabia⁴, the new find shows that by the end of the first half of the Miocene the distribution of hominoids extended almost the full length of the African continent (at this time the Arabian peninsula was geographically part of Africa). Soon afterwards, hominoids apparently dispersed out of Africa, the first record being from Paşalar, Turkey^{5,6}, dated 14–15 Myr. About three million years later they are known from the south of France in the west to Pakistan in the east, and they extended their range to China later in the Miocene. To some extent, this is paralleled by the present distribution of apes in Africa and south-east Asia, but whereas today the apes are almost entirely restricted to tropical forest, their Miocene distribution was rather more extensive.

Relationships

In accounting for this notable difference in distribution between past and present hominoids, the phylogenetic relationship of the fossil species is critical. Conroy

and colleagues assign the Namibian hominoid to a new genus and species, on the grounds that it exhibits a unique constellation of characters that differentiate it from other middle-Miocene hominoids. They suggest a number of possibilities for its relationships: it may be (1) the sister-group to all large-bodied hominoids; (2) sister-group to the African ape and human clade; (3) sister-group to the African ape clade; and (4) sister-group to the human clade. Because the evolutionary relationships of most previously known Miocene hominoids could be more precisely determined than that, the value of this discovery would be tempered by its very uniqueness, precluding its association with any other fossil group. But is the new specimen really that unique?

The basis for the uniqueness of the Namibian hominoid is provided by the 12 characters listed in the generic diagnosis by Conroy *et al.* on page 144. Five of them appear to support the idea that it is the sister-group to all large-bodied hominoids, but none at all can be found to support the other three possibilities (see the table accompanying this article). Several characters are of doubtful significance, and only two or three distinguish the Namibian hominoid from other fossil species.

Many of the characters, however, are shared with the older fossil hominoid *Afropithecus*, and it would have been interesting if the Namibian hominoid could have been compared with *Afropithecus* in this paper. More detailed examination may support the generic distinction, but on the evidence pub-

lished here it would not appear to be justified.

The species distinction, on the other hand, seems to be well founded, based as it is on an argument implicit in the paper. This is that, whereas many or most of its characters are present in other Miocene hominoids, there is none that shares them all, and it is the pattern of characters that is used as the species-defining complex. Le Gros Clark⁷ restricted his concept of total morphological pattern to functionally related characters and my own extension to this was to infer that a functionally related character complex may be a stronger phylogenetic tool than single characters taken in isolation⁸.

Principles

How do these principles apply to the new hominoid? The 'squared-off' molars (character 4 of the generic diagnosis in Conroy *et al.*) might relate functionally to the large retromolar space (characters 7 and 9), and to the large size of the second molar relative to the others (character 5). Such a combination might be related to the presence of thin enamel (but see ref. 9) and the little differential wear on the molars (characters 8 and 10), this pattern perhaps indicating a dietary function (eating of soft fruit) little different from that of *Proconsul*¹⁰. It would have been interesting to have had the authors' views on the significance of such a pattern, which may also be present in *Afropithecus* — although the Namibian hominoid is smaller than the type species of that genus, it is similar in size to the referred species *A. leakeyi* (= *Heliopithecus leakeyi* Andrews & Martin 1987) from Saudi Arabia.

It is a fascinating speculation that the furthest southwards extension of the hominoid range in the Miocene was phylogenetically linked with the furthest northwards extension of similar age. But speculation is all that it remains in the absence of a more rigorous taxonomic interpretation of this important discovery in Namibia. □

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ANALYSIS OF THE 12 CHARACTERS OF THE NAMIBIAN HOMINOID*

Characters supporting sister-group relationship with the great ape (orang utan, chimpanzee and gorilla) and human clade

- 1 'Puffy' molar cusps with Y5 pattern
- 2 Absence of beaded buccal cingulum
- 3 Presence of protostylar ridges on M₂ and M₃
- 6 Moderate size of inferior transverse torus
- 10 Little differential wear on molars

Characters probably ancestral for all hominoids

- 8 Molar wear indicating thin enamel
- 12 Narrow incisor region

Characters of unknown significance

- 7 Large retromolar space
- 9 M₃ not obscured by ascending ramus (same as 7)

Possible unique characters in the Namibian hominoid

- 4 Square molars
- 5 M₂ > M₃ > M₁
- 11 Even depth of mandible

Characters shared between the Namibian hominoid and *Afropithecus*

- 1, 2, 4, 6, 11, 12

* Numbers and characters refer to the list of Conroy *et al.* on page 144.

1. Conroy, G. C., Pickford, M., Senut, B., Van Couvering, J. & Mein, P. *Nature* **356**, 144–148 (1992).
2. Leakey, R. E. & Leakey, M. G. *Nature* **324**, 143–146 (1986).
3. Leakey, R. E. & Leakey, M. G. *Nature* **324**, 146–148 (1986).
4. Andrews, P. & Martin, L. *Bull. Br. Mus. nat. Hist.* **41**, 383–393 (1987).
5. Alpagut, B., Andrews, P. & Martin, L. *J. hum. Evol.* **19**, 397–422 (1990).
6. Bernor, R. & Tobien, H. *J. hum. Evol.* **19**, 551–568 (1990).
7. Le Gros Clark, W. E. *Fossil Evidence for Human Evolution* (Chicago University Press, 1955).
8. Andrews, P. in *Molecules and Morphology* (ed. Patterson, C.) 21–53 (Cambridge University Press, 1987).
9. Grine, F. E. *Palaeont. afr.* **28**, 61–69 (1991).
10. Kay, R. F. *Nature* **268**, 628–630 (1977).

Lighting up *Drosophila*

Peter A. Lawrence and Gines Morata

THOSE interested in animal and plant development have long cherished the ambition to describe exactly how, by what lines of descent, the cells of the embryo generate the cells of the adult. This aim has been achieved only in the nematode *Caenorhabditis elegans*¹. The difficulties are many; not the least is that, unlike *C. elegans*, most animals do not have an invariant pattern of cell lineage. Yet, even in those that do not there are constraints and rules that are well worth discovery, but the lack of good objective methods has barred progress. A paper by J.-P. Vincent and P. H. O'Farrell in the latest issue of *Cell*² describes a smart method for tracing cell lineage in living embryos that is, apparently, accurate yet does not interfere with the normal course of development. It has been tried out in *Drosophila* but may be applicable far more widely.

Much is known about the precursors of the adult cells of *Drosophila*, thanks to the genetic methods of marking cells that were developed mainly in the 1970s — these studies uncovered a link between cell lineage and the realms of action of a special class of controlling or 'selector' genes³. So far, direct studies of cell lineage in the early embryo have proved very difficult, which is unfortunate because it is precisely in the young embryo that the principles of genetic design are being worked out^{4,5}.

The new method requires the injection into the uncleaved egg of a 'caged' fluorescein (devised by Tim Mitchison). The caged fluorescein is not fluorescent and spreads throughout the egg, being taken up by each nucleus as it forms. Later, a single nucleus can be hit by an aimed beam of light which photoactivates ('uncages') the fluorescein, and when that cell divides it gives rise to a group of fluorescent daughter cells. The distribution of cells in this clone can then be compared, for example, with the sets of cells expressing some gene of interest. The fluorescent images are stored and "manipulated to enhance contrast" — probably the trickiest step in the process. The results, as shown in the *Cell* paper², are most impressive (see figure).

Vincent and O'Farrell have used this method to address an important question in *Drosophila* biology: what is the relationship between expression of the *engrailed* gene and cell lineage? Up to now this question has been approached only for the embryonic precursors of the adult cells, experiments that led to the current hypothesis for the role of the *engrailed* gene.

Tracking the cell lineage of the adult

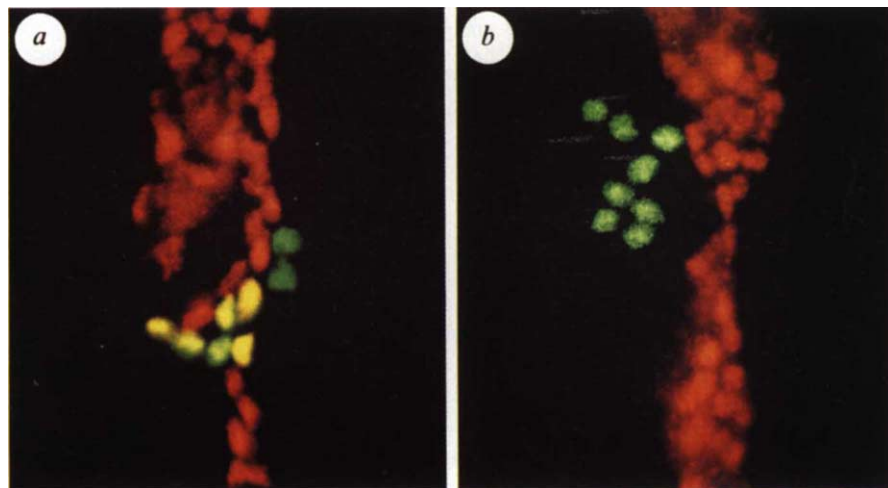
precursors revealed that the embryo contains sets of cells that are arranged in stripes, each stripe having the potential to generate a distinct part of the adult. The *engrailed* gene is only required in alternate sets, those that generate the posterior compartments. In the adult, both the expression of, and requirement for, *engrailed* are coextensive with the posterior compartments as independently defined by studies of cell lineage⁶⁻⁸. These earlier experiments also suggested that *engrailed* is needed to keep the groups of cells segregated from each other — without the gene, posterior cells come to lack some 'label' and can then mix with anterior ones. If all these observations are true, *engrailed* must be a key gene that acts to distinguish and deploy adult precursors in the embryo. Do these rules also apply to the embryonic cells that will make the larva?

Vincent and O'Farrell label cells at the blastoderm stage and study the marked clones 4 h later when nearly all the cell divisions have been completed. The clones constitute about 6–8 cells. The authors tested whether the two edges of the *engrailed* stripes, anterior and posterior, each delineate a cell lineage boundary. For the anterior edge, they find that clones of cells marked with the fluorescent label never straddle it and are always entirely on one side of the line or the other. By contrast, clones may cross over the posterior edge. So the anterior edge of the nascent *engrailed* stripe is a lineage boundary, whereas the posterior edge is not.

It had previously been proposed that the anterior edges of the *engrailed* stripes, which are collinear with, and probably defined by, the anterior edges of the *fushi tarazu* and *even-skipped* stripes, are lineage boundaries⁹. The new results show this to be true, at least over the period of the experiment.

The crossing of the posterior edges was not expected, however, as the posterior edges were thought to correspond to the segment boundaries. In *Drosophila*, the origin of the adult segment boundaries cannot be easily studied because larval cells intervene between the adult segmental primordia. In many other insects this is not the case and in one, the milkweed bug *Oncopeltus*, the segment boundary is both a lineage boundary¹⁰ and a boundary to *engrailed* expression¹¹, at least in postembryonic life. If we assume, as we like to assume, that both insects are built on similar principles, and therefore that the *engrailed* stripe, when mature, should define the segment boundary at its posterior limit, then Vincent and O'Farrell must be observing an immature and indefinite *engrailed* stripe.

In their paper they provide independent evidence for this. First, they show that the *engrailed* stripe narrows as a proportion of the segment's width and argue that this is to be explained by some cells that originally expressed *engrailed* not passing on the same property to all their descendants. Second, they hint that if similar blastoderm clones are looked at later in embryonic development, when the *engrailed* stripes have narrowed, they now rarely cross the posterior edges. It may be that the *engrailed* stripe narrows to include exactly all the cells on one side of a lineage



Uncaged fluorescein, used to map the expression of the *engrailed* gene in clones of *Drosophila* cells. a, A clone straddling the posterior edge of the *engrailed* stripe, demonstrating that, at this edge, there is no stable distinction between cells expressing and not expressing *engrailed* at the cellular blastoderm stage. Note that the clone does not cross over the anterior edge. b, Example of a clone not expressing the gene. The photographs are double exposures, with green showing a marked clone, red *engrailed* and yellow the coincidence of red and green signals.

Courtesy J.-P. Vincent

boundary, a boundary line that was formed independently of *engrailed*.

How do these incisive results affect our picture of the role of the *engrailed* gene? Clearly, *engrailed* is differently controlled in the anterior and posterior parts of the stripe; this does not fit with the simple view that *engrailed*, from the moment it is first activated, remains irrevocably switched on in sets of cells and all their descendants. Other recent results showing that the determination of *engrailed* expression is a complex and progressive business also point away from such a simple view¹². But the main picture of the *engrailed* gene survives these experiments. It was suggested that *engrailed* is responsible for both the posterior cell state and some cellular label that keeps the cells of different compartments segregated⁶. Vincent and O'Farrell's results with the anterior boundaries of the *engrailed* stripes are fully consistent with this view, but the instability of *engrailed* expression they observe at the posterior edge raises some new and interesting problems that have to be faced. What determines the seg-

ment boundaries? Are there pair-rule genes that develop sharp boundaries of expression and so define those boundaries? Are they located by gradients of positional information? And do the stripes of *engrailed* expression come to respect the segment boundaries precisely, even in the embryo? □

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1. Sulston, J. E. et al. *Dev Biol.* **100**, 64–119 (1983).
2. Vincent, J.-P. & O'Farrell, P. H. *Cell*, **68**, 923–931 (1992).
3. Garcia-Bellido, A. et al. *Sci. Am.* **241**, 102–110 (1979).
4. Nüsslein-Volhard, C. *Development* 1991 Suppl. 1–10 (1991).
5. Lawrence, P. A. *The Making of a Fly* (Blackwell Scientific, Oxford, 1992).
6. Morata, G. & Lawrence, P. A. *Nature* **255**, 614–617 (1975).
7. Kornberg, T. et al. *Cell* **40**, 45–53 (1985).
8. Hama, C. et al. *Genes Dev.* **4**, 1079–1093 (1990).
9. Lawrence, P. A. et al. *Nature* **328**, 440–442 (1987).
10. Lawrence, P. A. *J. Embryol. exp. Morph.* **30**, 681–699 (1973).
11. Campbell, G. L. & Caveney, S. *Development* **106**, 727–737 (1989).
12. Heemskerk, J. et al. *Nature* **352**, 404–410 (1991).

MATERIALS SCIENCE

Missing atoms and melting

Robert W. Cahn

IT is rare indeed for a scientific paper to remain central to current concerns several decades after its publication; in general, papers decay like last winter's leaves or this summer's pop songs, and scientists then instead cite the latest review paper. One of the rare survivors is Walter Kauzmann's 1948 paper, *The Nature of the Glassy State and the Behavior of Liquids at Low Temperatures*. The increasing number of physicists who are struggling to come to grips with that most familiar and most recalcitrant of processes, melting, keep coming back to Kauzmann and his eponymous paradox. The most recent author to collide with Kauzmann's paradox is Fecht, in a paper on page 133 of this issue¹. He has collided before², but this latest encounter is particularly fruitful.

The paradox (Kauzmann himself carefully called it "an apparent paradox") is simply that the equilibrium entropy of a supercooled liquid, when extrapolated, appears to reach a temperature, not far above absolute zero, at which it is lower than the entropy of the equilibrium crystal; this is absurd and it would then appear that such a liquid would be absolutely unstable and necessarily pass over continuously into the crystalline state, analogously to the continuous condensation of a compressed gas in the critical region. (This postulated critical behaviour was a popular notion in the

1930s, see Simon³.) The idea is a paradox because at these low temperatures atomic mobility is much too low to permit crystallization by any recognized mechanism. Such crystallization in the absence of the necessary atomic mobility is fashionably termed a 'catastrophe'. Kauzmann went on to resolve the paradox by pointing out that there must indeed be a continuous transition; it is not, however, a change from the equilibrium liquid to the crystal, but rather a congealing of the liquid to form a glass (which is simply a liquid that cannot remain in configurational equilibrium for lack of atomic mobility). The catastrophe has disappeared and an accepted transition has replaced it.

In their earlier paper² (which I discussed in these columns at the time⁴), Fecht and Johnson showed that a metal may have not only a lower isentropic temperature that limits the range in which an equilibrium liquid can exist, but also an upper isentropic temperature which marks the uppermost temperature at which a crystal metastably superheated above its thermodynamic melting point can survive the tendency to melt. This is again a kind of paradox derived from Kauzmann, because above this 'catastrophic' temperature, the crystal would have a higher entropy than the melt.

In that earlier paper, Fecht and Johnson pointed out the crucial role that

excess vacancies in the crystal must have on this upper limiting temperature. In the present paper¹, Fecht takes this argument a stage further. He shows how massive (nonequilibrium) vacancy concentrations reduce the normal thermodynamic melting temperature and eventually lead to a 'Kauzmann transition' well below the normal melting-point, T_m , of the perfect crystal.

By simply taking into account the standard excess free energy resulting from an excess concentration of vacancies, Fecht shows that the melting point drops with increasing vacancy concentration. Eventually a critical vacancy concentration, c_v^* , is reached, at which the slope of the plot of melting temperature versus c_v becomes infinite. The corresponding critical temperature, T^* , is that of the Kauzmann transition — a temperature of absolute crystal instability. For any pure metal, Fecht's computation shows that $c_v = 0.077$ and $T^* = 0.35T_m$. (For an intermetallic compound, c_v is the same but $T^* = 0.52T_m$.) It is not clear to me, however, that the possibility of a crystal with as much as 7.7 per cent of vacancies having a higher entropy than the corresponding melt really constitutes a paradox.

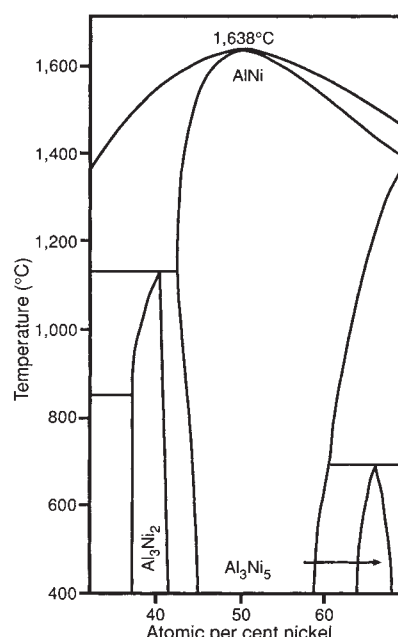
Fecht goes on to remind the reader of Gorecki's observation⁵ that during the normal melting transition (of a crystal with no more than equilibrium number of vacancies), the vacancy concentration at the (equilibrium) T_m rises from about 10^{-3} to about 10^{-1} ; accordingly, as Fecht points out, the Kauzmann melting of a crystal with about 8 per cent of vacancies amounts to a pseudo-critical conversion from crystal to melt without any significant change of vacancy numbers.

One can go on, beyond Fecht's arguments, to speculate what effect on melting might result from the presence of constitutional vacancies. These arise in non-stoichiometric binary compounds to avoid the greater thermodynamic evil of placing atoms of one species on the structural sites of the other (see my review⁶). Such vacancies were first discovered in the Al-rich range of NiAl. The large Al atoms cannot, it seems, occupy the sites of the small Ni atoms, and instead, increasing numbers of the Ni sites remain vacant as the composition diverges further from stoichiometry⁷. The latest, very precise measurement⁸ of the concentration of constitutional vacancies in NiAl fairly close to the Al-rich limit of the homogeneity range, at 54 atom-per-cent Al, is 7 per cent. (The concentration rises linearly with deviation from stoichiometry). If one now examines in the figure the shape of the solidus locus (that is, melting temperatures) for NiAl, one finds that the locus is highly unsymmetrical: it drops away much more steeply on the

ARCHAEOLOGY

The making of a mummy

Paul G. Bahn



Partial phase diagram for aluminium–nickel alloys⁸ (see text).

Al-rich side, where constitutional vacancies exist, than on the Ni-rich side, where there are none. Such a degree of asymmetry is rather unusual in equilibrium diagrams, and it may not be too fanciful to suggest that there could be a linkage between this feature and the presence of large numbers of vacancies.

Of course, the formation energy of constitutional vacancies is much smaller than that of normal thermal vacancies, and so the increase of the free energy of a crystal containing the former is much less (for a given concentration) than it is for the latter⁶. (The free energy is reduced by the presence of the vacancies, relative to a crystal with excess Al atoms in Ni sites, but is increased relative to the perfect crystal.) It might be worth examining more carefully the possible influence of very large concentrations of vacancies in certain compounds on their melting behaviour, particularly as Fecht's calculations show that the critical (Kauzmann) temperature for an intermetallic compound falls at a higher fraction of the normal T_m than it does for a pure metal. □

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THE lid has, it seems, finally been put on a controversy about whether the ancient Egyptians used bitumen when mummifying the dead. In their latest paper on the topic¹, J. Connan and D. Dessort show unequivocally that bitumen was indeed generally used in mummification, and far earlier than hitherto suspected, and they also pinpoint the most likely sources of the material.

The very word 'mummy' is derived from the Persian 'mumia' meaning bitumen or pitch, and the 'Mummy Mountain' in Persia was famous for the black, bituminous substance that oozed from it and was said to have medicinal properties². From the blackened appearance of many preserved bodies from ancient Egypt (see illustration), it was at first assumed that the corpses had been soaked in bitumen. In more recent times, however, Egyptologists have generally taken it to be the case that the corpses' appearance resulted from their having been coated with resins that eventually turned black³.

In the late 1980s, Connan and Dessort, both of whom are geochemists, analysed parts of an Egyptian mummy in the Musée Guimet, Lyon, and discovered traces of bitumen, the characteristics of which pointed to the Dead Sea as the source⁴. Together with similar results⁵, this indicated the use of bitumen in mummies from Ptolemaic and Roman times (about 400 BC to AD 300).

Connan and Dessort have now¹ found bitumen in a further dozen mummies dating from the reign of Ramesses II (about 1200 BC) to the Roman period, thus pushing the practice far back in time. Using petroleum geochemistry techniques, they have identified geochemical 'fossils' (steranes and terpanes) in hydrocarbon fractions of the balms which demonstrate that bitumen was generally an important ingredient in the embalming process. Moreover, on the basis of 15 discriminant molecular parameters, the authors have pointed to two principal sources. The first is again the Dead Sea, where asphalts occur in floating blocks. The other is the region of Hit-Abu Jir in Iraq, from where bitumen

used to be exported to Babylon for use as mortar in brick walls⁶.

The value attached to bitumen, reflected in the great distances from which it was imported, may lie simply in its supposed medicinal, antiseptic and preservative properties. But it is also possible that its use on corpses was linked to a belief in rebirth, which was symbolized by the colour black in ancient Egypt. The task that now remains for Connan

IMAGE
UNAVAILABLE
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REASONS

The black is bitumen — an Egyptian mummy of the Ptolemaic period (c. first century BC).

and Dessort is to see whether bitumen was also used on the very earliest mummies, those dating from 2600 BC to 1300 BC. □

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1. Fecht, H. J. *Nature* **356**, 133–135 (1992).
2. Fecht, H. J. & Johnson, W. L. *Nature* **334**, 50–51 (1988).
3. Simon, F. *Ann. Physik* **68**, 278 (1931).
4. Cahn, R. W. *Nature* **334**, 17–18 (1988).
5. Gorecki, T. *Scripta metall.* **11**, 1051–1057 (1977).
6. Cahn, R. W. in *Encyclopedia of Materials Science and Engineering* (ed. Bever, M. B.) 812 (Pergamon, Oxford, 1986).
7. Bradley, A. J. & Taylor, A. *Proc. R. Soc. A* **159**, 56–72 (1937).
8. Kogachi, M., Minamigawa, S. & Nakahigashi, K. *Acta metall. mater.* (in the press).

1. Connan, J. & Dessort, D. *C.r. hebdomadaire. Séances. Acad. Sci., Paris* **312**, 1445–1452 (1991).
2. David, R. & Tapp, E. (eds) *Evidence Embalmed: Modern Medicine and the Mummies of Ancient Egypt* 3 (Manchester University Press, 1984).
3. Lucas, A. & Harris, J. R. *Ancient Egyptian Materials and Industries*, 4th edn, 303–308 (Histories and Mysteries of Man Ltd, London, 1962).
4. Connan, J. & Dessort, D. *C.r. hebdomadaire. Séances. Acad. Sci., Paris* **309**, 1665–1672 (1989).
5. Ruilkötter, J. & Nissenbaum, A. *Naturwissenschaften* **75**, 618 (1988).
6. Connan, J. & Deschesne, O. *La Recherche* **22**, 152–159 (1991).

First light on fluid carbon

Nicolaas Bloembergen

RESEARCHERS at the University of Texas, Austin, may have caught the first, fleeting glimpse of fluid carbon. D. H. Reitze, H. Ahn and M. C. Downer describe in *Physical Review* (B45, 2677–2693; 1992) how both graphite and diamond can be melted momentarily by intense laser irradiation before expanding as a hot plasma.

The liquid phase of the element carbon is elusive, as it appears to exist in equilibrium only at temperatures of about 5,000 K and at pressures above several hundred atmospheres. The phase cannot be contained in any vessel, because all other materials melt or chemically react before the temperature required for the liquid state of carbon is reached. Clearly the structure of this state is of interest to those studying condensed matter physics, but because these conditions can be found in planetary interiors, it is also important for geophysics and astrophysics.

The first evidence for this phase came from experiments using pulsed ohmic

heating of graphite electrodes. Shock-wave studies and pulsed-laser heating of graphite and diamond have also been tried (see F. B. Bundy's review in *Physica* 156A, 169–178; 1989). Downer and colleagues used laser pulses lasting less than a picosecond (10^{-12} s) in their new experiments, both to create and to probe their fluid carbon.

It is well known that short, intense laser pulses are capable of turning any substance into a high-temperature, high-density plasma. By careful control of the energy fluence in the pump pulse it is possible to determine a threshold for melting as the first stage in the process of plasma formation. A second, probe pulse will be reflected or will generate second-harmonic (or frequency-doubled) light, and the change in either of these can be followed as a function of the pump fluence, the time elapsed, and the wavelength, polarization and angle of incidence of the probe. This parameterization gives detailed information on the changes in electronic structure of the

surface layer to a depth equal to the optical absorption depth and permits the determination of the material's dielectric function $\epsilon(\omega)$. Several detailed experiments with pico- and femtosecond pulses have been performed on silicon and gallium arsenide, as well as several metals over the past decade.

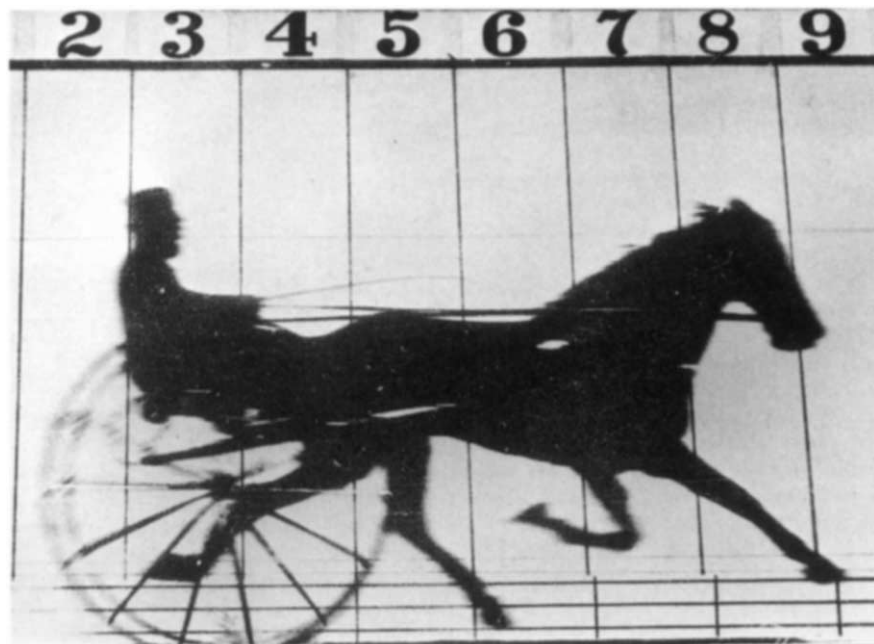
The pump-probe experiments of Downer and colleagues with a temporal resolution of about 0.1 ps reveal a sharp transition to a high-reflectivity phase at visible wavelengths, above a threshold, F_m , of the pump fluence. For graphite, $F_m = 0.13 \text{ J cm}^{-2}$; and for diamond, $F_m = 0.63 \text{ J cm}^{-2}$. What happens is that the pump pulse increases the number of mobile charge carriers in the graphite and then heats this electron plasma. This is confirmed by detailed analysis of the dielectric function for fluences below F_m .

Because the probe laser sees the electron plasma, not the state of the carbon ions, an indirect argument is needed to suggest that a fluid state of carbon is created. Indeed, for pump pulses above F_m , the phase transition initially is an electronic one, as it seems unlikely that there is enough time in the 0.1-ps pulse duration for the lattice of carbon atoms to be heated to the same temperature as the electron plasma. Instead, possibly a hot electron plasma coexists with relatively cold atoms. Although many of the electronic bonds responsible for graphite's rigid structure will have been broken, the atoms may still have a relatively small kinetic energy and have not yet had enough time to form the liquid phase. But in about 0.5 ps, thermal equilibrium may have been established between the hot electrons and the lattice, at which point the material will have become fluid.

The high reflectivity of the material lasts for a few picoseconds, but then drops. This is because of the expansion of the hot fluid, originally as dense as the graphite, into a rarefaction wave, during which the carbon-vacuum interface moves with a velocity in excess of the speed of sound. After 10 ps, the sharp boundary of the graphite is smeared over a distance comparable to one quarter of an optical wavelength (about $0.1 \mu\text{m}$), and the resulting gradient in refractive index reduces the reflectivity. Such effects have been seen previously with silicon and gallium arsenide heated above their critical points.

In diamond, the electron plasma is created by a three-photon absorption process across the large band gap between valence and conduction bands. This process requires a higher irradiance, which explains the higher threshold value of F_m . The diamond becomes opaque and the electronic structure appears to make a transition to the same fluid phase as observed with

Snap judgements



THIS remarkable photograph was taken by a remarkable man, Eadweard Muybridge, a pioneer of sequence photography. Muybridge — born Edward Muggeridge (he changed his name), died Eadweard Maybridge (a misspelling on his tombstone) — was English by birth but in 1851, at the age of 21, moved to the United States where he gained a high reputation as a photographer. Among his commissions was one from Leland Stanford, a former governor of California, to photograph a trotting horse and settle the controversy as to whether all four hooves are lifted off the ground at the same time. The answer, revealed by images such as that reproduced here, was that they are. The work of Muybridge and others is celebrated in an exhibition which opened this week at the Museum of the Moving Image in London. Entitled "Catching the Action: Muybridge and the Chronophotographers", it runs until 31 May. **T.L.**

graphite. Equilibrium between the hot electrons and the carbon atoms is again established in about 0.5 ps. The initial fluid density now equals the density of diamond rather than of graphite, and the internal pressures are very high — of the order of 100,000 atmospheres.

One of the interesting questions about fluid carbon is its electronic state: is it semiconducting like diamond, metallic like graphite (in the planes) or insulating? The reflectivity data, determined partly by the electron plasma and partly by bonding valence electrons, suggest the d.c. resistivity is 650 $\mu\text{ohm cm}$, so that fluid carbon could be described as a poorly conducting metal.

The data do not permit a more detailed description of the phase's structure. It would be interesting to probe the phase by other techniques, for example by X-ray diffraction with sub-picosecond time resolution. The dielectric function indicates there is substantial valence bonding, so that perhaps the fluid phase contains molecular fragments such as C_2 or C_3 , which X-rays could reveal.

It is instructive to compare the fem-

tosecond experiments on carbon with those on other materials. These all exhibit a well defined fluence threshold, F_m , for melting, but there exists an interval, typically between F_m and $2F_m$, in which the liquid phase is stable and not susceptible to hydrodynamic expansion. For fluences larger than $2 - 5F_m$, these other materials get heated above their boiling point and their critical point on a timescale shorter than one picosecond. Only then do these materials undergo the expansion that is unavoidable in carbon. In the case of GaAs, whose crystal structure lacks a centre of symmetry, the abrupt disappearance of second-harmonic generation by the probe laser for pump fluences above a sharp threshold proves the sudden transition to a fluid phase before rarefaction or evaporation can take place. But with symmetric graphite or diamond, second-harmonic generation is not an appropriate probe. □

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MOLECULAR TRANSDUCTION

Do the ear's links link?

Jonathan Ashmore and Michael Evans

OF ALL families of ion channels, those activated by mechanical forces remain perhaps the most elusive. The difficulty has been that, unlike channels such as the sodium or acetylcholine receptor channels, there just are not very many of them about. More importantly, the molecular nature of the channel perturbation and its coupling to cell deformation may not be the same in all mechano-sensitive cells, a class which includes cells as varied as vascular endothelia, sensory receptors and oocytes. Writing in *Neuron*¹, Assad *et al.* provide further hints about how the conversion of mechanical into electrical energy occurs, at least for hair cells in the inner ear.

Hair cells are found in all peripheral structures used in hearing and balance. The cells get their name from the bundle of specialized, pivoting microvilli, termed stereocilia, projecting from their apical surface. As mechano-sensors the cells are extremely sensitive: nanometre deflections of the hair bundle are sufficient to produce a signal which is then relayed up to the central nervous system. But they are sensitive to deflection of the hair bundle in one direction only (towards the tallest stereocilium) and all explanations of mechano-electrical transduction must be able to take this into account.

Although most data are derived from

lower-vertebrate hair cells, it is generally agreed that the mechano-electric conversion step is too fast to be explained by known enzyme or second-messenger systems. The best available electrophysiological data indicate that the transduction channels are located near the tips of the stereocilia², even though earlier work using calcium imaging and exploiting the permeability of the transduction channels to calcium had suggested that the channels were located much closer to the base of the bundle³ (a result that now looks increasingly unlikely). Further, but circumstantial, evidence for a channel at the tip is the presence of a fine extracellular filament, possibly elastin, running between the top of each stereocilium and its taller neighbour⁴. These filaments have been termed 'tip links'.

A model for hair-cell transduction which combines these features is therefore a type of trapdoor-and-rope arrangement⁵. In this scheme, the tip link is connected to the channel so that bending the bundle tensions the link, opening the channel (see figure). This is a seductive hypothesis to explain transduction in all types of hair cell; it is consistent with some measurements of the forces required to move the bundle and explains why, because of the link orientation, the bundle has to be stimulated in one direction.

Pedigree whiskers

NOT so long ago, synthesizing diamonds was a tough task, requiring heat and high pressure. But in the mid-1980s it turned out that diamond will settle out of thermally decomposed hydrocarbon gas. To rub in the simplicity of it all, researchers then learned that welders unwittingly make diamond crystallites in the flames of their oxyacetylene torches. P. W. Morrison Jr, J. E. Cosgrove and P. R. Solomon now exploit this process to make synthetic-diamond filaments (*Appl. Phys. Lett.* **60**, 565–567; 1992). Guided by a microscope, Morrison and colleagues drew out of their torch flame tiny filaments up to 1 mm long by 0.25 μm across. In principle, the authors report, there is no limit to the length of the filaments, which could outclass normal carbon fibres. But the growth rate of under 200 $\mu\text{m h}^{-1}$ may discourage entrepreneurs.

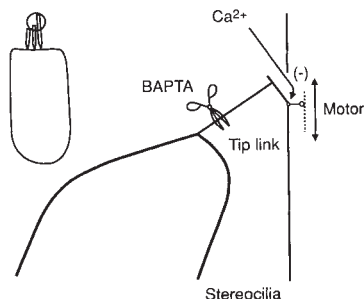
Sociable beetles

BETTER now join the ants, termites and mammals (the African mole rat) on the list of taxa with eusocial members (D. S. Kent & J. A. Simpson *Naturwissenschaften* **79**, 86–87; 1992). The weevil *Austroplatypus incompertus* lives in galleries in the heartwood of *Eucalyptus* trees. Colonies are initiated by solitary fertilized females and, when mature, manifest the three phenomena which characterize eusociality: overlapping generations, cooperative brood care and division into reproductive and sterile (unfertilized) castes. Each colony contains one fertilized and five or so unfertilized adult females, the job of the second group being to deal with predators and to extend and maintain the galleries. Two related species of weevil live in the heartwood of other trees: one is not eusocial, but nothing is known about the social life of the other.

NMR PDQ

PROTEIN structure determination by NMR is no easy way to earn a living. But for the many who would be satisfied with less than a high-resolution crystal structure, D. S. Wishart *et al.* (*Biochemistry* **31**, 1647–1651; 1992) show how much can be achieved with remarkably little effort. They have devised a simple routine for assigning α -helical and β -structure to the sequence of any protein small enough to give a sharp proton NMR spectrum. Their method rests on the observation that the α -CH signal of each amino acid shifts upfield when it enters the α -helical state and downfield when it is in the β form. The α -CH shifts in the two-dimensional spectrum of the protein are then marked as +1, 0 or -1, and three or more successive resonances shifted in the same direction denote a segment of the ordered structure. All the indications are that the method is foolproof; Wishart *et al.* aim next to add criteria for turns.

Single transduction channel openings have been measured in hair cells, but only with difficulty: the number of active channels must be reduced drastically by a transient exposure to micromolar levels of calcium⁶. Transduction is abolished irreversibly by the treatment. The experiments described by Assad *et al.* provide an explanation for this manipulation by showing that the tip links can be removed physically by treating the bundle with very low levels of calcium (using BAPTA as a chelator for 10 seconds). More remarkably still, the



A model of hair cell transduction in the stereocilia (site circled on hair cell in the inset). The tip links run between the top of one stereocilium and its neighbour, where the tensioning motor could be sited. The channel may be at either end of the link. A route for calcium entry is shown. The links are severed by BAPTA¹.

bundle moves slightly (by 10–100 nm) towards the tallest stereocilium as though the bundle had previously been under tension. These observations show that there are ways of altering components of the bundle very selectively.

The question of whether the links are directly coupled to the channel or to some other intermediate structure remains unclear. Is it possible that the calcium-sensitive tip links do nothing more than simply hold the bundle together, and that the mechano-electric transduction step occurs where adjacent membranes of the stereocilia come into contact? Oocyte stretch-activated channels⁷, the hair-cell transducer and apical membrane channels from epithelial cells are sensitive to amiloride, which blocks channels in a voltage-dependent manner. Surprisingly, there is an antibody which binds to the latter two but which does not necessarily co-localize to either end of the hair-cell tip link⁸. This might even indicate that there are more channels in the apical membrane than those associated with the tip links.

In hair cells, channels opened by bundle movement eventually close (although they can be reopened by further displacement), such adaptation depending upon the internal concentration of calcium^{9,10}. So what is the interaction between calcium and the tip link? In order to re-exert a force on the channel after a steady displacement, the link needs either to shorten or to reposition

itself along the stereocilia. One proposal is that a molecular motor placed at one end of the link pulls the filament tight⁵. Even though there are suggestions that the motor may be fuelled by ATP¹¹, it would have to possess some curious properties: it would need to run continuously but to slip, and to slip more with increased levels of internal calcium in order to reduce link tension. Transmembrane sliding-motor systems are known in flagella¹², but are not as yet described in sensory cells.

The other explanation for hair-cell adaptation is that the transducer channel is itself closed by the calcium which enters. This would form a feedback loop tightly regulating the activity of the channel¹⁰. But although this model accounts for many features, it does not explain the loss of transduction under micromolar calcium conditions. The problem with the tip-link model is that it predicts changes in bundle stiffness not seen during experimentally controlled adaptation¹⁰. There are thus features of the transduction process which neither explanation can entirely account for.

Assad *et al.* nonetheless describe the first observed relationship between bundle morphology and transduction currents since hair-cell signals were shown to be produced by deflection of the stereocilia of the hair bundle¹³. Their measurements of bundle motion may even hint at a rapid tensioning of the links, before they snap. It should make matters much clearer when the forces in the tip links are measured directly. The next stage is to dissect the components of the transducer channel to determine its precise molecular environment. The problem is that there are only about 100 channels in each hair cell: now, there's a challenge for molecular biology. □

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1. Assad, J. A., Shepherd, G. M. G. & Corey, D. P. *Neuron* **7**, 985–994 (1991).
2. Jaramillo, F. & Hudspeth, A. J. *Neuron* **7**, 409–420 (1991).
3. Ohmori, H. *J. Physiol.* **399**, 115–138 (1988).
4. Pickles, J. O. *et al.* *Hearing Res.* **15**, 103–112 (1984).
5. Howard, J., Roberts, W. A. & Hudspeth, A. J. *Rev. Biophys. biophys. Chem.* **17**, 99–124 (1988).
6. Crawford, A. C., Evans, M. G. & Fettiplace, R. *J. Physiol.* **434**, 369–398 (1991).
7. Lane, J. W., McBride Jr, D. W. & Hamill, O. P. *J. Physiol.* **441**, 347–366 (1991).
8. Hackney, C. M. & Furness, D. N. *J. Physiol.* **438**, 124P (1991).
9. Assad, J. A., Hacohen, P. & Corey, D. P. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2918–2922 (1989).
10. Crawford, A. C., Evans, M. G. & Fettiplace, R. *J. Physiol.* **419**, 405–434 (1989).
11. Gillespie, P. G. & Hudspeth, A. J. *Biophys. J.* **61**, 516a (1992).
12. Bloodgood, R. A. in *Prokaryotic and Eukaryotic Flagella* (eds Amos, W. B. & Duckett, J. G.) 353–380 (Cambridge University Press, 1982).
13. Hudspeth, A. J. & Jacobs, R. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1506–1509 (1979).

Evil spirits

THE hangover, that stern natural punishment for alcoholic overindulgence, is blamed not on alcohol itself, but on the various chemical 'congeners' present in most alcoholic drinks. Aldehydes, tannins, acids and higher esters are the chief villains. They are most plentiful and ferocious in chemically complex drinks like brandy and red wine. Those who mix their drinks, and thus get a richer assortment of congeners, are said to suffer worst.

Daedalus is pondering these strange facts. Congeners, he notes, cannot make up more than a few parts per thousand of any drink — yet they can temporarily induce a degree of malaise and wretchedness normally experienced only by the seriously ill. More wonderful still, they do it after about an eight-hour delay. DREADCO chemists are therefore scouring the market for the cheapest red plonk, the crudest brandy and the most debased 'firewater' whiskies, and are extracting their congeners. Mixed together and concentrated to about a ten per cent solution, the congeners will form a subtle and horrific liquor. The hapless consumer won't even get drunk. But a few hours later he will get the father and mother of all hangovers.

With a little chemical cunning "Head-splitter" (as this fearsome beverage is known among its creators) may even be made quite palatable. For the flavour and character of a drink are also due to its congeners. The DREADCO team is being supervised by a sadistic wine-master, who hopes to endow Headsplitter with a rich and appealing bouquet.

The plan, of course, is to market Headsplitter as a classic form of aversion therapy for otherwise unreformable alcoholics. The patient (victim might be a better word) will be offered a generous glass of the product; a few hours later, when it is just about to strike, he will be given a little of his favourite tippie. The devastating effect, and its apparent cause, should make an indelible impact on even the most dedicated connoisseur.

Headsplitter may find wider markets. Many states impose only four criminal punishments: fine, imprisonment, death, and confiscation of driving licence. Headsplitter could provide a fifth sanction. Cheap, easily calibrated and administered, and doing no lasting harm, it could transform jurisprudence. Yet Daedalus suspects that even this grisly deterrent may acquire devotees of its own. One theory of alcoholism holds that it is a self-destructive 'game' whose unacknowledged payoff is the hangover. Headsplitter will deliver a payoff of unparalleled intensity, and a black market may develop in the product. David Jones

'Peacock' viscous fingers

SIR — Viscous fingering is a growth phenomenon resulting in intricate patterns that arise from the Saffman–Taylor instability¹, manifested when a low-viscosity fluid displaces another with a higher viscosity. Although the phenomenon has been known for many years, the ability of these fingers to develop fractal patterns has only recently been realized². Recently, at the Hotel Los Lebreros in Sevilla, Spain, I came across an example of viscous fingering over much greater length and timescales than I had ever seen before.

According to the waiters, the history of the 'experiment' is as follows. Several years ago the hotel coffee room was closed off with a large anticrack window divided into several 5×5 m panes. Each pane was made up of three parallel glass plates separated from each other by a very thin film of polyvinyl. The upper and lower edges of the three glass sets were sealed to a frame by a layer of silicone (Fig. 1), while the edges were sealed by the same material to adjacent anticrack window panes. But several small holes in the paraffin covering the window edges allowed air to penetrate the base, gaining access to the polyvinyl between the glasses. Thus, an unplanned but potential Hele Shaw cell³ was created, the result being an example of what we would call "dynamic fractal self-decoration": after a few months, some fingers appeared in the window. The fingers later split, creating a branched structure and today, 11 years later, three beautiful fractal fans up to 35 cm in radius, which the waiters call "the peacocks", decorate the coffee room of the hotel (see Fig. 2).

This example of viscous fingering is interesting for reasons other than curiosity and beauty. First, there is no doubt that the process is triggered by temperature. This is supported by the fact that

the pattern is restricted to the outer film, that is, the one that takes the brunt of the climate and is unprotected by the air conditioning inside the hotel. In addition, those window panes not facing the street are unaffected. The explanation can be provided by considering that the affected window panes are oriented toward the south and sunshine in Sevilla can cause the temperature of the outer film to rise above 60 °C. This temperature must be higher than the threshold temperature at which the polyvinyl film becomes a viscous fluid. Under these conditions, the window becomes a perfect Hele Shaw cell, in which two mechanisms can account for the generation of these fractal patterns. One is the invasion of air through an initial hole pushing the viscous thin film and the other is the progressive separation of the glass plates due to thermal constraints, which is known to produce a dielectric breakdown pattern⁴. The perfect semi-circular envelope of the fractal pattern can be explained by the interaction of both mechanisms. To my knowledge, this is the first evidence of a thermally activated Saffman–Taylor instability.

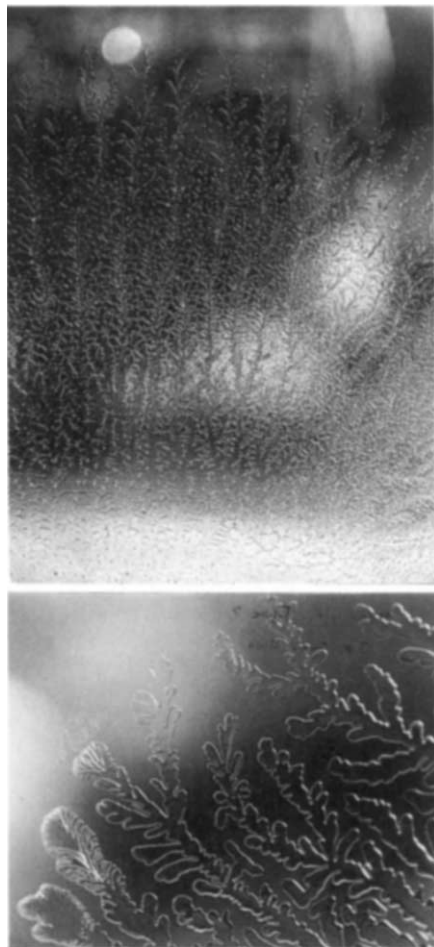


FIG. 2 Top, view of the 'peacock' pattern; bottom, detail of the fingering.

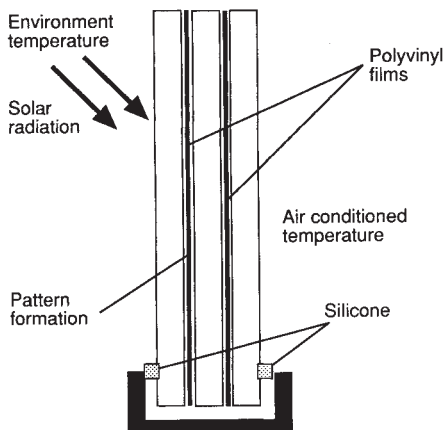


FIG. 1 Cross section of the anticrack window.

Second, the 'peacock' patterns at Los Lebreros represent an example of a large-scale viscous-fingering experiment. One can calculate the mean velocity of the tip propagation to be about 0.1 mm per day. So, the viscous film should be immiscible with air, because the characteristic length of the fluid displacement is similar to the characteristic diffusion length, although no diffusion patterns are observed. Even more surprising is the fact that this Hele Shaw cell should work discontinuously because the thermally driven viscoplastic behaviour of a polyvinyl film is a reversible phenomenon. Accordingly, during the night, when the temperature does not exceed 30 °C, the process is dormant. The same can also be expected for longer periods during the winter. This intermittency could be the source of the banding structure observed.

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1. Saffman, P. G. & Taylor, G. *Proc. R. Soc. A* **245**, 312 (1958).
2. Nittmann, J., Daccord, G. & Stanley, H. E. *Nature* **314**, 141 (1985).
3. Hele Shaw, H. J. S. *Nature* **58**, 34 (1898).
4. Niemeyer, L., Pietronero, L. & Wiesmann, H. J. *Phys. Rev. Lett.* **52**, 1033 (1984).

CFTR mechanism

SIR — In his recent News and Views article¹, Wine discussed evidence^{2,3} that expression of the cystic fibrosis transmembrane conductance regulator (CFTR) carrying the common $\Delta F508$ mutation in non-epithelial cells results in appearance at the cell membrane of protein with chloride-channel activity. The new data showed that channel activity could be stimulated by a mixture of cpt-cyclic AMP, forskolin and the methylxanthine IBMX.

We had previously reported⁴ that IBMX can stimulate CFTR function. We have since found that the action of IBMX is unlikely to be mediated by effects on cellular cyclic AMP levels⁵. The actions of methylxanthines, in particular Ca^{2+} mobilization or adenosine receptor antagonism, should be investigated in the development of drug strategies for the treatment of cystic fibrosis.

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1. Wine, J. J. *Nature* **354**, 503–504 (1991).
2. Dalemans, W. et al. *Nature* **354**, 526–528 (1991).
3. Drumm, M. L. et al. *Science* **254**, 1797–1799, (1991).
4. McPherson, M. A. et al. *Lancet* **ii**, 1007–1008 (1986).
5. McPherson, M. A. & Dormer, R. L. *Molec. Aspects Med.* **12**, 1–81 (1991).

Optical thickness of galaxies

SIR — Burstein, Haynes and Faber¹ claim to have demonstrated, for the first time, that spiral galaxies are optically thick at the faint isophote of 25th blue magnitude arcsec⁻² ($25 B_{\mu}$). Apart from the astrophysical difficulties of such a dramatic hypothesis, which Burstein *et al.* do not address, the claim itself is spurious because they neglect a well-known selection effect which can naturally explain all the trends in their 16 separate diagrams, even if all their galaxies are entirely transparent.

The test for opacity they are using is a minor variant of Holmberg's longstanding surface-brightness/inclination test², to which the addition of distance information is largely irrelevant as both surface brightness and inclination are distant-independent quantities. What Burstein *et al.* have discovered in their data, as Valentijn³ did earlier, is that the measured surface brightnesses $\Sigma_m \equiv 1_{ap}/\pi ab$ of their galaxies are remarkably constant, that is, within $\pm 1.5 B_{\mu}$, a result made explicit in their Fig 6.

There are two different interpretations of that result. Either the sample galaxies have been unconsciously selected to have a constant surface brightness, independent of inclination, in which case nothing whatsoever can be deduced about their opacity from the Holmberg test. Or the data are unaffected by such a selection effect, in which case their galaxies are indeed opaque at $25 B_{\mu}$ where the diameters were measured.

But it is well known³⁻⁵ that a sample of galaxies chosen for their apparent size, such as the UGC sample used by Burstein *et al.*, is subject to very strong surface-brightness selection effects so that most objects in it, irrespective of their optical depths, will have measured Σ_m s clustered within ± 1 mag of a certain optimal value. This is because lower surface brightness galaxies hide so much of their light below the sky that they look small at a given isophote, whereas higher surface-brightness galaxies are, at a given luminosity, intrinsically and isophotally smaller. This striking effect, which acts independently of inclination⁶, renders Holmberg-type tests useless for determining optical depth.

It is up to Burstein *et al.* to retest their data and demonstrate that they are not explicable in this straightforward way. Readers of scientific papers can usually

make such internal consistency checks for themselves. Unfortunately, although it purports to be a review, Burstein *et al.*'s paper is almost wholly based on what the authors themselves describe as a 'private database'. A paper based on 'hidden' data places itself above the normal processes of scrutiny that distinguish science from polemics.

Some results in science are harder to believe than they are to prove and the surprising effects of surface-brightness selection on galaxies may fall into this category. It is difficult, particularly for seasoned observers, to believe that they dominate almost all the discovered correlations between the global properties of galaxies, but they do.

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BURSTEIN *ET AL.* REPLY — It is not sufficient to note, as Disney does, that two parameters (surface brightness and inclination) are distance-independent. We showed¹ that the major factor in determining the results of various workers is sample selection, and sample selection has traditionally depended on the distant-dependent parameters of apparent magnitude and apparent diameter. Choloniewski has separately demonstrated this fact⁷. Our method is fundamentally different from Holmberg's² because we use the distance itself, in the form of the redshift, to break the former degeneracy between apparent and intrinsic quantities for galaxies. Indeed, our method cannot use surface brightness as part of the test.

If very low surface-brightness (VLSB) galaxies are excluded completely from these catalogues, our results naturally apply only to the higher surface-brightness galaxies that are included. In this case, our result applies only to the given sample, which is all we have claimed. If, as Disney hypothesizes, VLSB galaxies are preferentially present as small-appearing galaxies in the ESO and UGC catalogues, they would affect our sample only if they had different inclination-dependent properties than those of higher surface-brightness galaxies. We agree that such an effect would preferentially affect the smaller appearing galaxies. That is one reason why we divide our sample into different ranges of apparent diameter. The fact that the result of inclination dependence of absolute diameter does not change with the apparent diameters of the galaxies would imply that significant contamination by VLSB galaxies has not affected our results for these higher surface-brightness galaxies.

Redshifts for most of the UGC galaxies used in our sample come primarily from published papers and catalogues (although our compilation of these data is private). ESO-LV data were used for our Fig. 6 (surface brightness versus inclination), as the edge-on galaxies in the UGC generally have unreliable magnitudes.

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No diabetes link to hsp65?

SIR — Jones *et al.*¹ suggest that the diabetes-susceptibility gene *IDD5* may encode the mouse heat-shock protein 65 (hsp65), citing their previous report² indicating that hsp65 is a β -cell antigen in insulin-dependent diabetes (IDD) in man.

Although such a link between hsp65 and IDD is attractive, the role of hsp65 as an autoantigen in IDD is by no means as generally accepted. Following the original report of Jones *et al.*², two further reports^{3,4} indicated that the autoantigen in IDD was distinct from hsp65 on the basis of size, antibody reactivity and tissue distribution. Jones *et al.* subsequently indicated⁵ that the critical result in their original report may have been due to the presence in the IDD sera of antimycobacterial antibodies. Moreover, a well-documented paper⁶ identified the autoantigen in IDD as the *M*_r 64,000 protein glutamic acid decarboxylase rather than hsp65.

Any assessment of the significance for diabetes of the similar chromosomal localization of *IDD5* and an hsp65-homologous gene must await good evidence that hsp65 is an autoantigen in human IDD. It may be of significance, however, that the *Lsh/Ity/Bcg* gene, which determines susceptibility to various infectious bacteria, also maps to this region of chromosome 1 (and is possibly identical to *IDD5*, ref. 7) as the main human immune response to these infectious agents is directed against their homologues of human hsp65 (ref. 8).

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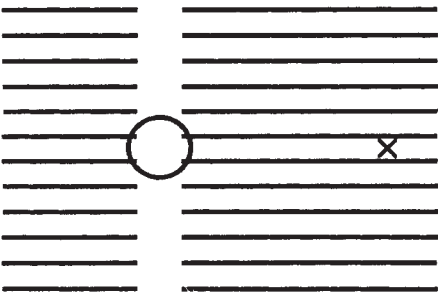
1. Burstein, D., Haynes, M. & Faber, S. *Nature* **353**, 515–521 (1991).
2. Holmberg, E. *Medd. Lund. Ser II* No 136 (1958).
3. Valentijn, E. *Nature* **346**, 153–155 (1990).
4. Disney, M. *Nature* **263**, 573–575 (1976).
5. Disney, H. & Phillips, S. *Mon. Not. R. astr. Soc.* **205**, 1253–1265 (1983).
6. Davies, J. I. *Mon. Not. R. astr. Soc.* **244**, 8–24 (1990).
7. Choloniewski, J. *Mon. Not. R. astr. Soc.* **250**, 486–504 (1991).

1. Jones, D.B. *et al.* *Nature* **355**, 119–120 (1992).
2. Jones, D.B. *et al.* *Nature* **336**, 583–585 (1990).
3. Kampe, O. *et al.* *Lancet* **336**, 1250 (1990).
4. Atkinson, M.A. *et al.* *Lancet* **336**, 1250–1251 (1990).
5. Jones, D.B. & Duff, G. *Lancet* **337**, 115 (1990).
6. Buckleskov, S. *et al.* *Nature* **347**, 151–156 (1990).
7. Cornall, R. J. *et al.* *Nature* **353**, 262–265 (1991).
8. Kaufmann, S.H.E. *Immun. Today* **11**, 129–136 (1990).

Filling in the blind spot

SIR — Andrews and Campbell¹ described an ingenious experiment on the blind spot². Does 'filling in' of the blind spot involve creating an explicit representation³ of the surrounding colour or pattern, or do we simply ignore the absence of signals from this region — just as we ignore the back of our head?

To find out, we placed a yellow ring or doughnut (outer diameter 8°; inner diameter 4.5°) on the blind spot so that its inner margin fell entirely inside the blind spot. The ring then looked like a homogeneous yellow disk, being 'filled in' by the yellow from the ring. Furthermore, when we had several rings scattered randomly in the visual field, with one ring alone being positioned on the blind spot, then the latter 'popped out'



This display schematically illustrates a vertical illusory strip that passes through the subject's blind spot (circle) as he fixates the X with his left eye. Interestingly, it is the illusory contours that get 'completed' rather than the real horizontal lines that define them.

preattentively even in naive subjects, that is, it was conspicuously visible as a disk against a background of rings. Because pop-out implies early visual processing, we conclude that the filling in also occurs relatively early. Also, our results suggest that one really does 'fill in' the blind spot — people do not just ignore what is out there. For how can something being ignored 'pop-out'?

We also find that filling in is a sensory rather than cognitive process. A line traversing the blind spot is seen as complete but if a circle (diameter 10°) falls partially on the blind spot, the visual system does not complete the arc of the circle; it looks 'chopped off'. Hence, filling in occurs at the level of surface and contour interpolation rather than the level of object representation.

Finally, if a vertical illusory strip is made to pass through the blind spot, then the visual system completes the illusory contours across the blind spot instead of completing the horizontal lines that define the illusory contours (see figure). Again, if the visual system were simply ignoring the blind spot, it is

hard to see how the observer could make this discrimination.

The recent experiments of Gilbert and Wiesel⁴ support our conclusion that the filling in observed in this display is an active process that occurs early in visual processing³. Gilbert and Wiesel destroyed a small patch of retina (in cats and monkeys) and recorded from cells in the corresponding area of visual cortex. These cells were initially silent, of course, but within a few minutes the same cells could be excited by visual stimuli that lay outside the scotoma⁴. Next, instead of destroying retinal receptors, Gilbert and Wiesel repeated these experiments using a display similar to the one we used for generating the artificial scotoma. To their surprise, they found that cells which initially had receptive fields within the square now had receptive fields that were much larger and included regions outside the square⁴. Because these cells were originally responding to stimuli inside the scotoma, perhaps higher brain centres are 'fooled' into thinking that stimuli immediately outside the scotoma are

now actually inside it and this would explain the perceptual 'filling in' effects that we observed.

Furthermore, these results imply that the classical receptive field may be just the tip of an iceberg. Each cell may have thousands of silent synapses that can be reactivated or inhibited — perhaps in seconds — in response to visual stimulation, this can manifest as dynamic changes in receptive field organization. Studying these changes may provide insights about the neural mechanisms underlying perception.

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1. Andrews, P. R. & Campbell, F. W. *Nature* **353**, 308 (1991).
2. Brewster, D. *Letters on Natural Magic* (Murray, London, 1832).
3. Ramachandran, V. S. & Gregory, R. L. *Nature* **350**, 699–702 (1991).
4. Gilbert, C. & Wiesel, T. N. *Soc. Neurosci. Abstr.* **17**, 1090 (1991).

■ See the Letter by C. D. Gilbert and T. N. Wiesel on page 150 of this issue.

Apolipoprotein B intermediates

SIR — We showed that, unlike a conventional secretory protein, the amino-terminal 15% of apolipoprotein B (apo B) translocates the endoplasmic reticulum membrane via transient, transmembrane intermediates before conversion to the fully translocated form¹. Because these intermediates are wholly extractable from the bilayer at pH 11.5, we suggested that apo B pauses (stops and restarts) in the aqueous translocation channel at several points, perhaps to allow modification, assembly or regulation before secretion. Pease *et al.*² disagree with our findings and our model of apo B biogenesis.

The most important difference is that we find intermediates with progressively longer amino-terminal lengths protected from exogenous proteases which become fully protected over time; Pease *et al.* find only the latter fully protected forms. They believe our findings are due to over-proteolysis, whereas we believe that they have failed to detect transient intermediates. Thus, we propose that the translocation of apo B proceeds stepwise until it is fully protected, whether it is completely in the lumen or associated with the membrane of the endoplasmic reticulum. Pease *et al.* believe that apo B is cotranslationally inserted into the inner leaflet of the membrane, bypassing any transmembrane translocational intermediates. These issues might have been simply resolved by an exchange of materials and discussion of protocols which we offered through our correspondence with *Nature*.

A second way to resolve the dispute is to test the two models. One model predicts that translocation of apo B pauses at multiple, specific sequences within the protein, and that such pausing can be dissociated into stopping and restarting, which would simplify detection of the otherwise transient intermediates. The other model predicts that translocation does not pause and hence, sequences with this function do not exist in apo B.

We have now³ (1) identified a 'pause transfer' sequence early in apo B which causes translocation of a defined heterologous chimaeric protein; (2) dissociated that pause into distinct stop and restart steps; (3) demonstrated another pause transfer sequence in its native context later in the protein suggesting that multiple such sequences are involved in the biogenesis of apo B; and (4) described a method to keep a transient transmembrane intermediate stopped for up to 2 hours before restarting its translocation post-translationally, rendering it readily detectable even under the experimental conditions of Pease *et al.* We believe these data will resolve the controversy and will allow progress in the study of this unusual phenomenon.

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PEASE *ET AL.* REPLY — The report by

Lingappa and colleagues¹ of transmembrane intermediates with tetracaine suggested to us² that overdigestion may have occurred and that the processes described might be occurring inside the membrane. Although tetracaine does not inhibit the proteolysis of proteins spanning the lipid bilayer^{2,4}, it could alter the structure of the translocation pore and affect proteolysis of proteins anchored in the pore, the topology that Lingappa and colleagues now envisage for apo B^{3,5}. We have therefore carried out reactions with tetracaine present throughout the translation reaction, and find that it does not inhibit protein synthesis, processing or glycosylation. This does not seem consistent with tetracaine causing a gross structural change in the translocation pore.

Chuck and Lingappa have now described a protocol for trapping presumptive intermediates of translocation³. They say that this method is reproducible either with wheat germ or rabbit reticulocyte lysates; proteinase K or trypsin; and Ca²⁺ or tetracaine — that is, it fulfils the criteria we would have expected for demonstrating a transmembrane structure with large cytoplasmic domains⁵. Transcripts lacking termination codons are translated, generating proteins covalently linked at their C terminus to transfer RNA, which in turn may remain tethered to ribosomes^{4,6}. In this configuration, the ribosomes may remain bound to microsomal membranes with the translocation pore jammed open.

Chuck and Lingappa claim that some truncations reveal transmembrane domains, while others do not. For example, an intermediate is trapped between residues (1 → 400) but not residues (1 → 304) or (1 → 471), which they interpret as a pause signal between 304 and 400 with a restart signal between 400 and 471. If the concept of successive pause and restart signals is correct, it is easy to explain the complete translocation of apo B17 translated with a stop codon as we observed². We find it harder to understand, on the basis of Lingappa's current model, why apo B15 with a stop codon should have paused at multiple sites in the work reported in ref. 1, however, as if successive restart signals were unrecognized by the translocation apparatus. In our opinion, the latest data of Lingappa and colleagues do not distinguish between the 'trapping' of transmembrane intermediates due to absence

of downstream restart sequences and interference of the normal process of translocation by the transfer RNA moiety. We believe that the apparent translational pausing observed by Lingappa and colleagues may be secondary to translational pausing, which we find can be greatly increased with transcripts lacking stop codons.

Although we received an offer of exchange of reagents from Chuck and Lingappa in a letter to us in October 1991 after publication of our paper, we did not perceive such an offer in correspondence we received from *Nature*.

We still believe that cotranslational insertion into the inner leaflet of endoplasmic reticulum is probable. Whether

or not there are transient discontinuities in translocation that can be revealed (or produced artefactually) by *in vitro* translation, we have seen no evidence for the stable post-translational transmembrane intermediates under the conditions reported in ref. 1.

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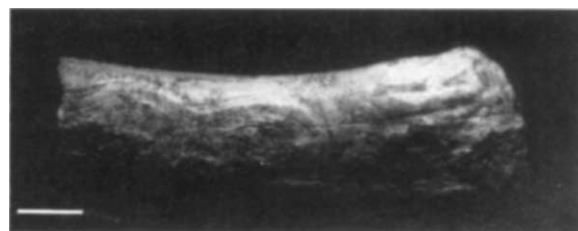
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Palaeolithic art found in China

SIR — A decorated antler fragment has recently been recovered from a 13,000-year-old upper palaeolithic occupation layer in Longgu Cave near Xinglong, Hebei Province, China¹. It bears three distinctive, carefully engraved and complex non-iconic patterns. Traces of a thick ochre coating are partly preserved under calcium carbonate encrustation. The 14-cm-long object was fashioned from antler of *Cervus elaphus canadensis* and weighs 104 g. A small amount of its spongy interior has been dated by accelerator mass spectrometry. The radiocarbon age of 13,065 ± 270 before

artificially perforated stone disk, presumably a pendant. At 28,135 ± 370 years BP, it is one of the earliest drilled stone objects in the world. About 120 perforated objects previously found in the Upper Cave at Zhoukoudian, near Beijing, are more recent. Palaeolithic disk beads made from ostrich eggshell, similar to those found in India³ and Africa, occur in the Gobi Desert as surface finds.

The antler fragment, the first object of palaeolithic art found in China, adds weight to the hypothesis that most graphic art of the Pleistocene is non-figurative or 'geometric'⁴. Outside the southwest of Europe, true two-dimensional representational depiction is almost entirely lacking in palaeolithic art. In Asia, only two instances of it are known³. Even in the Franco-Cantabrian region, figurative representations are outnumbered about three times by non-



Palaeolithic antler fragment from Longgu Cave, Hebei Province, China. Scale bar, 2 cm.

present (BP) confirms that of the occupation layer.

One of the three engraved designs consists of four sets of six or seven parallel wave lines, the other of an elaborate figure eight motif (see figure) while the third pattern is an arrangement of parallel and zigzag lines enclosing two elongate panels of oblique crosshatching. The bold layout of all three designs and the excellent workmanship suggest the hand of a highly experienced artisan, backed by a sophisticated tradition of producing such non-representational art.

Previous reports of portable palaeolithic art in China have been refuted. Examination of a sample of markings on some 600 bone fragments from Shiyu, Shanxi Province², has shown that they are the result of several taphonomic processes¹. However, the upper occupation layer of that site has yielded an

figurative art.

Moreover, pleistocene art is found in all continents except Antarctica, and its beginnings may predate the Aurignacian of Europe in two or three continents. Therefore the attention given to the western European art body, at the expense of all other arts of roughly similar antiquity, is scientifically inexpedient and has created a distorted picture of early art development. It accounts for one of several strongly established biases in this area of research.

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1. Bednarik, R. G. & You Yuzhu *Rock Art Res.* **8**, 119–23 (1991).
2. You Yuzhu *Kexue Tongbao* **29**, 80–82 (1984).
3. Bednarik, R. G. *Proc. 23rd Chacmool Conf.* (1991).
4. Bednarik, R. G. *Zeitschr. f. Ethnol.* **112**, 223–35 (1987).

1. Chuck, S. L., Yao, Z., Blackhart, B. D., McCarthy, B. J. & Lingappa, V. R. *Nature* **346**, 382–385 (1990).
2. Pease, R. J., Harrison, G. B. & Scott, J. *Nature* **353**, 448–450 (1991).
3. Chuck, S. L. & Lingappa, V. R. *Cell* **68**, 1–13 (1992).
4. Knight, A. M., Harrison, G. B., Pease, R. J., Robinson, P. J. & Dyson, P. J. *Eur. J. Immun.* (in the press).
5. Lingappa, V. R. *Cell* **65**, 527–530 (1991).
6. Perara, E., Rothman, R. E. & Lingappa, V. R. *Science* **232**, 348–352 (1986).

Continuing conversations

Donald Michie

The Cybernetics Group. By Steve J. Heims. MIT Press: 1991. Pp. 334. £22.50, \$33.75.

It is the fate of science's inside stories to be chronicled from the outside. When, very rarely, an insider goes public, as James Watson did in *The Double Helix*, peers view it as an act of indiscretion or worse. Yet books like Watson's have a particular and compelling merit. Along with the gossip, the reader knows he is getting the scientific goods. Heims, on the other hand, spares the content, ever-impatient to plunge into action and the personalities — their differences, their reconciliations, their letters to each other and above all their conferences.

The reader is duly warned in the first chapter:

Studying the interactions of such clusters . . . offers a good counterweight to the sort of history one would write solely on the basis of published research results. Indeed, it is my view that such result-oriented history misrepresents the nature of these sciences.

The book accordingly preoccupies itself with attitudes, causes, issues, personal philosophies and the like. True to the proclaimed stance, it omits results. Heims is concerned to describe "a moment when a new set of ideas impinged on the human sciences . . ." These new ideas came to prominence during and just after the Second World War, and offered analogies between sociobiological systems and machines. Looking back, Warren McCulloch wrote: "Then it was that the dream began of team play between biologist, mathematician and communication engineer, which eventually flowered into cybernetics . . ."

Half a century later, how has the team play worked out? Assessments of a sort are offered in the closing chapter. Heims clearly feels that all those conferences paid off. Does the reader perhaps suppose that the computer revolution, with its manifest impact on society, developed from work to be traced through the computer science literature? Wrong. We have it from Heims that it developed from ideas aired at US multidisciplinary conferences sponsored between 1946 and 1953 by the Macy Foundation:

An abundance of technological developments and mass production of devices and systems of communication and computation grew out of the ideas reported by the cyberneticians at the Macy meetings. It became the mainstream of high technology in America and elsewhere in the ensuing decades.

Computer scientists and technologists

of course acknowledge John von Neumann for his chairmanship of the early post-war EDVAC committee for the design of a digital computer with a stored program. But this came his way not because he was a cybernetician (a term yet to be invented) but because he was von Neumann. Computation's highest representative body, the Association for Computing Machinery, has made several awards. Not one ever went to a cybernetician. By what route, then, did the Macy meetings engender the compu-



Man and machine: von Neumann and 'stored program computer'.

ter revolution? Heims does not say.

There is an irony here. Human factors, a latter-day branch of the cybernetics movement, is now coming to the fore in the computer world. The descent from cybernetics is not in doubt. In his newly published second edition of *Engineering Psychology and Human Performance*, Christopher Wickens traces it

. . . from the new language of information theory and cybernetics that after World War II began to replace the stimulus-response language of behavioral psychology as a description of human activities. Terms such as *feedback*, *channel capacity*, and *bandwidth* began to enter the descriptive language of human behavior. This new language enabled some aspects of human performance to be described in the same terms as the mechanical or electronic systems with which the operator was interacting.

As workstations and personal computers proliferate, design of ever more human-like interfaces has become a competitive commercial art, drawing heavily on human factors as well as on software technology and applied artificial intelligence. Does Heims rejoice that a cybernetical offshoot finds a place? Visiting the Mecca of such work, he records that the Media Lab at the Massachusetts Institute of Technology

. . . brings sophisticated computer systems to bear on such technical problems as

creating a "receptionist" machine that can recognize individuals and respond appropriately, sensibly, and helpfully in all situations. It has a section dealing with electronic publishing responsive to individual readers' interests. It seeks to develop computers that can respond to nonverbal communications such as the direction of a person's glance and the tone of his voice . . . No wonder the commercial sector is eager to support it. It is a boon for them.

Heims's orientation is left-liberal with a whiff of Rousseau. I sense in him a deep ambivalence towards machines and indeed to the industrial world as a whole:

Also missing from this electronic future are closer relationships to a natural non-human environment — mountains, oceans, trees, animals, small farms.

Heims ends his book by announcing a second coming: "A network of people

has formed in this new cybernetics tradition; their names appear repeatedly at conferences and in volumes containing articles on self-organization." The reader may not previously have encountered either the names, or the network, or the volumes. I certainly had not. So it would have been useful to have references to these. Instead, we have to be content with an announcement of the completion of a PhD thesis with which, anticlimactically, the book closes. The student, it seems, "explores in mechanical terms the means for a system to evolve new sensibilities, such as the capability of constructing or discovering hitherto unknown observables." With no pointer to the university where this thesis is lodged, we must presumably await publication. Meanwhile, to quote the book's final sentence, "the conversation continues".

Heims wrote an outstanding earlier book on John von Neumann and Norbert Wiener. The disappointing quality of the sequel arises, I think, from a number of factors. The stature of the earlier book's two intellectual heroes underwrote Heims's focus on human detail and social trends. Additionally, von Neumann was central to two post-war developments of geopolitical consequence, namely the digital computer and the Cold War. This offered appropriate openings for Heims's messianic gifts and a challenge to his powers

of liberal-humanist criticism. But having in the first book seen off in style his two leading players, he has wanted somehow to pull together the remaining cast into a separate production. The task was not really feasible.

Heims's manner of falling short, however, cheats the reader of a legitimate expectation. Rather than assurances that cybernetics still lives, the reader may prefer particulars of the accomplishments of its lifetime and an

introduction to its true intellectual heirs — today's fast-growing neurosciences and linked developments in the computer simulation of neural processes. The heritage owed to the cybernetics movement could have been Heims's focusing theme. It may perhaps provide the theme of an inside story still to be written. □

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Telling differences

Stuart Sutherland

Perceptual and Associative Learning. By Geoffrey Hall. Oxford University Press: 1991. Pp. 300. £30, \$45.

ASSOCIATIONISM, the doctrine that all mental events (and the ensuing behaviour) can be explained in terms of the association of ideas, was first systematically propounded by David Hume. At some level it must be correct: after all, the operation of the nervous system is governed by the connections between its cells. The real issues are what can be associated with what and how the associations are determined. Until recently, most workers in animal learning, such as Clarke Hull and B. F. Skinner, have assumed that the only associations made were between stimuli and responses (S-R theory) and that the strength of an association depended on the number of times reinforcements were given. Both assumptions have been discarded. Nowadays, all agree that connections between stimuli can be formed (S-S learning), and that, even if a reinforcer always follows a stimulus, no connection will be made if the reinforcer occurs as frequently in the absence as in the presence of the stimulus.

Geoffrey Hall concentrates on S-S learning: he aims to show that certain perceptual phenomena can be explained in terms of the associations used in animal-learning theory, a takeover bid that might have brought upon his head the resources of the Securities Council had he made it in the City (of London). His main target is a form of "perceptual learning" in which, under certain circumstances, organisms can learn to distinguish two stimuli to which they initially give identical responses; as a preliminary, he deals with a variety of other phenomena, including latent inhibition, the process by which repeated exposure to a stimulus reduces the stimulus's capacity to form associations.

Hall's arguments are based on many experiments, and are so detailed, subtle

and variegated that they cannot be summarized here. As a simple example of his theorizing, consider his account of "perceptual differentiation". A subject exposed to two stimuli subsequently shows an improved capacity to differentiate between them — for example, to learn one response to one, and a different response to the other. Hall's account depends on several assumptions, among them being that a stimulus is made up of "stimulus elements", that when an organism is exposed to a stimulus, salient elements acquire more latent inhibition than less salient ones, and that ability to discriminate between two stimuli is greater the higher the proportion of all stimulus elements not shared by the two stimuli. Hall postulates that each stimulus initially contains three different kinds of stimulus element: set 1, those not shared with the other; set 2, stimulus elements common to both stimuli; set 3, novel elements, which Hall assumes are also common to both stimuli. Because the novel elements are, according to Hall, the most salient, the latent inhibition generated by exposure will reduce their power to form associations more than it reduces that of the stimuli in sets 1 and 2. By reducing the novel elements, which are shared by both stimuli, pre-exposure will increase the proportion of stimulus elements that are specific to one or other stimulus. Hence, Hall claims, organisms will now discriminate between them more easily. Because novelty is not a property of the physical stimulus and must be detected by some such process as failing to match some elements of the stimulus to a stored representation, it seems odd to talk of "novel stimulus elements". Perhaps this does not affect Hall's argument.

Oddly, he does not extend his analysis to the phenomenon that many workers in visual perception regard as the most interesting example of perceptual learning. If someone views a range of similar objects such as cathedrals, he may at first find it hard to distinguish them, but with sufficient exposure he learns not only to distinguish those he has seen, but can on encountering a new example learn enough about it to tell it apart from all the others after a comparatively brief inspection. Hall could argue that all

the "common elements" of cathedrals have been reduced in associative power by the previous exposures, making the features unique to particular examples more salient, so that discrimination becomes easier. But if the person is made to give a verbal response to common features, for example to say "flying buttress" or "triforium", there should be no latent inhibition and these features will remain salient: the differentiating features will then have no advantage and perceptual learning should not occur, an outcome that seems unlikely, particularly as attaching names to the parts seems to help recognition of the whole. The standard account of this type of learning given by workers on perception is that it is at first difficult to form a detailed representation of a structure as complex as a cathedral because there is too much new information. Once the appropriate representations are formed, however, much of the perceptual input can be mapped onto them. To construct an adequate representation of a new cathedral, all that is necessary is to modify the existing general representation in the few ways needed to specify that cathedral uniquely.

Such is Hall's ingenuity that he might well find a way of twisting this account into the language of latent inhibition, common stimulus elements and so on. But if animal learning is to take over perception, it must face such problems as which stimulus elements are common and which are not. Indeed, the use of the vague and entirely abstract expression "stimulus element" may be regarded as a retreat to mental atomism as put forward by John Locke. Animal-learning theorists take for granted the processing of both stimulus and response, and in thinking of associations, they make no use of the concept of hierarchy, without which no account of perception or indeed of any other aspect of cognition can be given. It may be partly for this reason that theories of animal learning, despite their liberation from the S-R approach, still seem rather impoverished.

Despite these qualms, Hall's book is a *tour de force*: it convincingly explains a wide range of phenomena, even if the nearer it gets to perception the less convincing it becomes. Hall is totally honest in reviewing both evidence and theories: he does not attempt to disguise inconsistencies in the former nor ambiguities in the latter. He suggests enough new experiments to keep workers in animal learning busy for years. And he exhibits great zest: one might say that he writes with faith, hope and clarity. □

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Thorny controversies

Fred S. Rosen

Metchnikoff and the Origins of Immunology: From Metaphor to Theory.

By Alfred I. Tauber and Leon Chernyak. Oxford University Press: 1991. Pp. 236. £35, \$45.

Le Dernier Langage de la Médecine: Histoire de l'immunologie de Pasteur au Sida. By Anne Marie Moulin. Presses Universitaires de France: 1991. Pp. 447. FF220.

MORE than any of his contemporaries, Ilya Metchnikoff (1845–1916) has remained an appealing scientific figure a century after his work flourished. Intelligent, sensitive, neurotic to the point of being suicidal, and principled in his disdain of the tsarist bureaucracy, he ultimately found his fulfilment and a sense of optimism after his Hegira to Messina in Italy. There, by the Sicilian shore on a Saturday afternoon in December 1882, he inserted bits of a rose thorn into some starfish larvae and found that they were ingested by mesodermal cells that later came to be called phagocytes. This moment in the history of science is of the same genre as Archimedes in the bathtub or Newton under the apple tree; it appears to be a moment of brilliant insight unconnected to any past endeavour.

Not so. Tauber and Chernyak have explored in some excruciating detail the origins of Metchnikoff's experiment and once again have shown the truth of Pasteur's wisdom, that chance favours the prepared mind. For two decades before the Messina experiment, Metchnikoff had been deeply involved in the study of comparative embryology and the digestive function of embryonic cells. The acrimonious scientific debates with Haeckel and other German progenitors of embryology over problems of gastrulation paved the way for the more monumental struggle between the cellular and humoral advocates of immunity that was to be engendered by the discovery of phagocytosis.

Tauber and Chernyak have produced a scholarly and detailed study of the origins of the deep and long controversy between the cellular and humoral basis of immunity; it does not provide light reading. The exegesis of the controversy continues unabated because it is important to understand its origins — it set back the progress of the science of immunology by many decades. In the end, everybody almost had it right. On the way to understanding the basis of immunological specificity, those who

thought deeply about the subject became enmeshed in complex metaphysical ideas, from Metchnikoff and Ehrlich to Medawar, Burnet and Benacerraf. In the end one has to fault Metchnikoff for not considering the basis of immunological specificity. It is comforting to speculate that, had he done so, much of the acrimony would have been assuaged. The recent frenzied activity and elegant work on antigen presentation is a direct intellectual heritage of the ideas about intracellular digestion first laid out by Metchnikoff; certainly he would be pleased by the recent discoveries of how intelligently phagocytes behave.

Moulin has produced a more light-hearted volume on the history of the controversy. Although she claims to be

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Metchnikoff — Nobel prizewinner in 1908.

concerned with the semiotics of immunology, she has, in fact, produced a straightforward history of the subject. It does not have the scholarly depth of Arthur Silverstein's recently published book on the same subject (*A History of Immunology*, Academic Press, 1989); it is more appropriate for scientists in other fields or for the lay public. The subtitle "History of Immunology from Pasteur to AIDS" is misleading, because Moulin says little about Pasteur and even less about AIDS. Instead, she provides a charming discussion of the characters involved in the evolution of recent immunology and an illuminating look at their contributions to the field. It is tempting to say that a profound exposition of a subject can be produced only by a worker in the field, but *The Eighth Day of Creation* and such other well-written scientific histories belie that costive assertion. Our present ignorance of the immunopathogenesis of AIDS makes it certain that further volumes will be forthcoming before we are completely enlightened about the mechanism of immunity. □

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Wedded to calamity

Allen H. Perry

Natural Hazards. By E. A. Bryant. Cambridge University Press: 1991. Pp. 294. £40, \$79.50 (hbk); £14.95, \$29.95 (pbk).

THERE are many books now on natural and environmental hazards. Despite its boast, this one is not the first to offer an "inter-disciplinary approach which concisely and clearly explains how natural hazards occur and analyses specific case-studies of major historical and recent disasters". It is hard to judge just to whom this academic, coffee-table-format book will most appeal. The fly leaf says lay readers, students and professionals, but I feel that these groups will find the book less than satisfactory for their purposes. Although well illustrated with maps, diagrams and pictures, this is hardly a work of popularization. With its world-view, albeit with an Australian perspective, it is probably too sweeping a survey for most serious students of the field. Bryant does, however, provide good introductory reading lists of main papers and books at the beginning of each section, as well as an extensive glossary of terms to help the nonspecialist reader.

The arrangement of the chapters into three main sections — climate hazards, geological hazards and social impacts — perpetuates the old-fashioned and artificial division of hazard types. Land instability, for example, often arises from climatic extremes. It is also to be deplored that Bryant treats the effects of different hazards on individuals and societies quite separately from the causes, physical mechanisms and frequencies of the main hazard types. Indeed, too little attention to society-environment relations may often be the cause of hazards in the first place. At a time when a more holistic attitude to hazard studies is rightly gaining ground, this text seems to lack integration. Another unfortunate effect of the divisional approach is that it implies that hazards are, as K. Hewitt put it, "accidental rather than characteristic features of the places and societies where they occur, somehow roped off from the rest of man-environment relations". Such an attitude is established early in the first chapter when we are told that "this book is concerned foremost with the various ways that nature can wreak havoc upon humans".

To suggest, in light of today's unprecedented level of environmental change, that humans have little influence in turn-

Mary Evans

ing extreme events into hazards is surely to be naive. Bryant's conclusion that "when a natural disaster occurs . . . it is surprising that so few people die or are injured", also suggests that his optimism outweighs his judgement. Has he forgotten about the heavy death tolls in countries such as Bangladesh that have been caused by tropical storms and sea surges?

I cannot say that I share Bryant's optimism, not even for his book. Given the choice of texts on the subject, I would steer readers in other directions. □

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Putting on the agony

A. W. Duggan

Defeating Pain: The War Against a Silent Epidemic. By P. D. Wall and M. Jones. Plenum: 1991. Pp. 300. \$24.95.

The Culture of Pain. By David B. Morris. University of California Press: 1991. Pp. 375. \$29.95

ALTHOUGH a fair amount is known about how nerves signal damage and how the spinal cord processes this information, there is little understanding of how the brain handles the information to give pain perception. Any perception is associated with comparisons by the brain of past and present and it is axiomatic that cultural influences must be powerful in such comparisons. On this much, the authors of both these books are agreed. The approaches to pain taken by these two books, however, differ in many ways.

Wall and Jones set out to demystify pain. They are highly critical of the training of medical staff to see pain solely as an aid to diagnosis and not as a symptom requiring treatment in its own right. They argue that in many cases of intractable pain the cause either cannot be defined or cannot be eliminated and that, in these cases pain management should be made the top priority. In particular, repeated surgery purporting to remove causes of pain comes in for heavy criticism. The authors emphasize that the treatment of pain by simply attempting to interrupt a pathway from periphery to brain, either pharmacologically or surgically, is inadequate. Their main message for sufferers from chronic pain is that pain need not be tolerated and that sufferers should see themselves as active participants in its management.

Roughly 100 pages are devoted to

RADIO days — Advertisement for an RCA add-on car radio. In the United States, the car radio became a familiar fixture in the 1930s, with more than one million of them being sold in 1935. This picture is taken from Michael Brian Schiffer's *The Portable Radio in American Life*, which traces the cultural and technological history of the appliance from the mid-1890s, through the golden age in the 1920s, to the waning days of the "transistor revolution" in the 1960s. Published by the University of Arizona Press, price \$45.



explaining what is known about the scientific basis of nociception and pain. This section is liberally sprinkled with both clinical and everyday examples of pain and its paradoxes. Drugs used to treat pain are briefly dealt with; again the information to patients is not to fear drugs if they provide some relief. In an age when people are all too ready to condemn the use of pharmaceutical drugs, such advice is in itself a relief.

The other procedures used in pain management are so numerous that they are difficult to describe succinctly. Manipulation, nerve stimulation, acupuncture, surgical interventions, psychotherapy and the placebo effect all get due consideration.

Attempting to get a message across both to the general public and to health professionals is difficult, but in this task the authors of *Defeating Pain* can claim a large degree of success.

In contrast to Wall and Jones, Morris has few kind words to say about modern science. Indeed, he lays much of the blame for the epidemic of patients suffering from pain squarely at the feet of those who have taken an approach to pain based on anatomy, biochemistry and pharmacology and have neglected the cultural significance of pain. One of Morris's main conclusions is that pain would not be the cause of so much suffering and economic loss in Western society if its meaning in a cultural setting were appreciated by those who treat it. These are strong words, and although the author does call on many instances that illustrate how differing societies have viewed pain in differing contexts, I felt by the end of the book that this was an unproven hypothesis, albeit interesting.

Morris deals with the Christian approach to pain and suffering not only through early writings, but also through paintings and sculpture. The concept of Christian redemptive pain received a battering from the Marquis de Sade, and

Morris's interpretation of the writer's work as resulting from the mechanistic approaches of enlightenment medicine in the eighteenth century is skilfully developed.

The chapter on the "uses of pain" is rather disappointing, because Morris deals only with satire, torture and the use of pain to assert power and the pain of athletes and dancers. In fact, pain as a determinant of behaviour enabling an animal to survive is likely to be its main usefulness, but Morris is clearly employing the word "use" in the sense of a human inflicting pain or making a living through writing about pain.

In discussing tragedy as an art form, Morris seems happy to link suffering associated with pain perception with that from other causes. Suffering is difficult to define, but it may be regarded as a state resulting from the inability of an animal to interact with the environment in the accustomed (learned) way. Chronic pain is one cause of suffering, but so is isolation, starvation or immobilization. Whether the end result — appreciation of suffering in consciousness — is due to the same brain process is unknown, but (dare I say it) neuroscientists would like to think that ultimately that question can be answered.

The author's condemnation of what he calls the organic model of pain is rather overdone. To state that such thinking has been successful in silencing or subjugating all other cultural discourse on pain is nonsense.

Nonetheless, all those interested in pain would benefit from Morris's wide reading of both the general and scientific literature on the subject. He has many perceptive comments. Try not to be put off by his aversion to science. I, for one, thoroughly enjoyed reading the book. □

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Mammalian phylogeny: shaking the tree

Michael J. Novacek

Recent palaeontological discoveries and the correspondence between molecular and morphological results provide fresh insight on the deep structure of mammalian phylogeny. This new wave of research, however, has yet to resolve some important issues.

ALTHOUGH several major lineages of fossil and extant mammals are recognized, all of the more than 3,000 living species are members of the monotremes (comprising only the duck-billed platypus and the echidna), the metatherian (marsupials) or the eutherian (placental) mammals. This basic tripartite division leaves unclarified the relationships among a bewildering array of marsupial and eutherian lineages. Recently, this problem has been investigated using new systematic methods^{1,2}, a growing molecular database³⁻⁵ and continuing palaeontological discoveries⁶⁻⁸. Current work mirrors a long history of forays and setbacks in untangling higher mammalian relationships⁹, culminating in George Gaylord Simpson's classification of the Mammalia¹⁰ in 1945 (Box 1). The influence of this classification not only reflected Simpson's authority in the field but also his role as one of the architects of the modern synthesis¹¹, the melding of darwinian evolutionary theory with genetics and palaeontology that emerged in the middle of the twentieth century.

Refinement of Simpson's arrangement largely applied to generic and familial level taxa. An exponential increase in knowledge of fossil mammals¹², as well as developing molecular techniques, laid the groundwork for the current attention to the broader outlines of mammalian phylogeny. Molecular approaches were rooted in studies¹³ contemporaneous with

Simpson's classification and eventually were related to phylogenetic reconstruction¹⁴. Techniques evolved through immunological comparisons^{15,16}, protein sequencing^{17,18} and, most recently, direct comparisons of DNA sequences in selected genes^{5,19-21}. New systematic methods, such as cladistics^{2,22,23} and powerful computer programs^{24,25}, have allowed for analyses of diverse anatomical characters and nucleotide sequences under the same logical framework.

Morphological and molecular discrepancies

This conformity in method prompts an essential question: to what extent do phylogenies based on morphological traits either conflict or comply with molecular results? Given the limitations both in molecular^{19,26,27} and in morphological^{23,28} approaches, it is not surprising that higher mammalian phylogeny is still unclear (Fig. 1). For example, there is only limited congruence among various data sets²⁹. Some morphological traits²⁹ and protein sequence data^{18,30} offer strong support for Simpson's Paenungulata, the superorder which includes the living hyraxes, elephants and sirenians (sea cows and dugongs)³¹. Nonetheless, a group of morphologists³² dispute the association of hyraxes with other alleged paenungulates and favour a pre-Simpsonian idea³³ that hyraxes and perissodactyls (the order including modern horses, rhinos and tapirs) are very close relatives. This

BOX 1 Simpson's 1945 classification of mammals

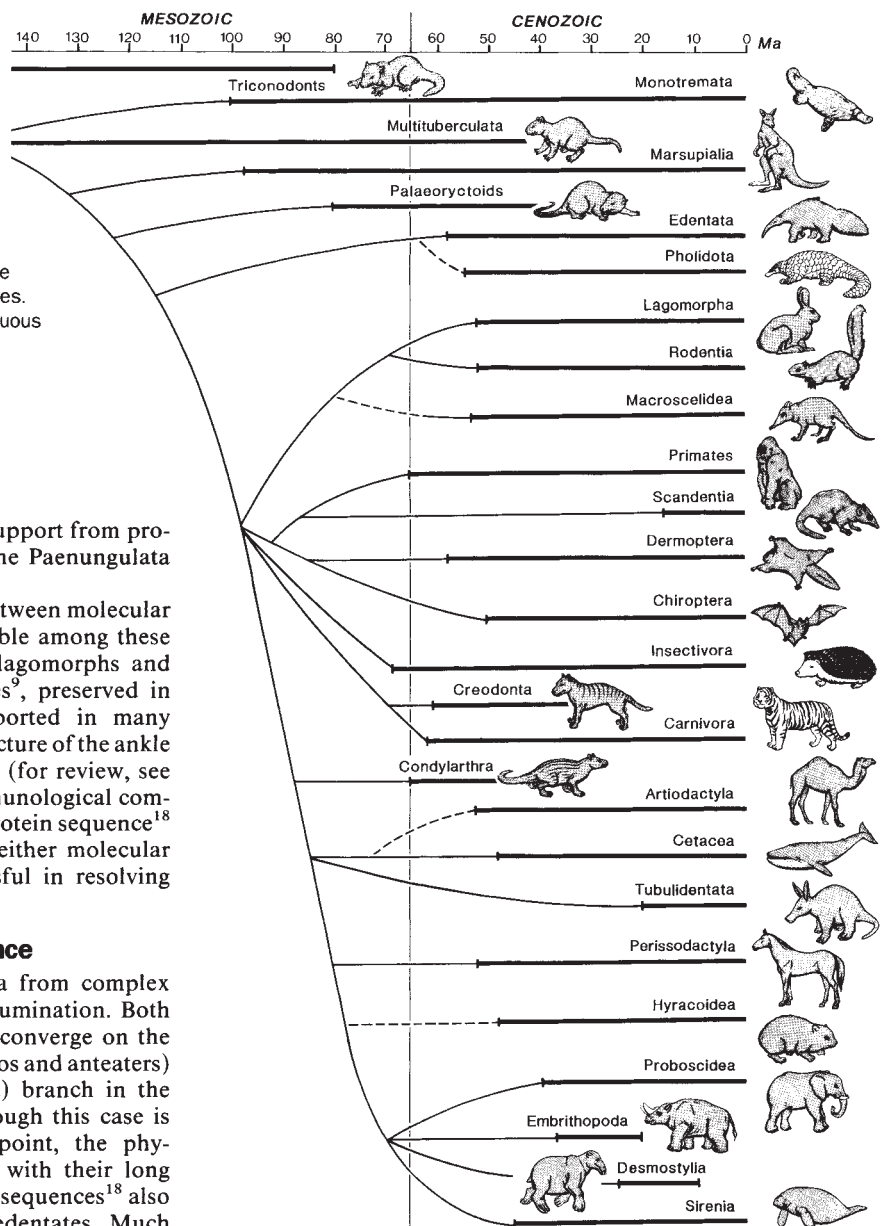
Simpson's classification had two primary objectives: to outline the author's version of the principles of systematics and to illustrate the application of such principles with an exhaustive catalogue, down to the generic level, of the living and fossil mammals. Genera, families and orders were arranged under several cohorts and superorders. Some of these higher categories were drawn from earlier work of W. K. Gregory⁹ and others. A few of these concepts, such as the grouping of carnivore and ungulate orders within the Ferungulata, were audacious and original. Moreover, they were heavily influenced by information on early fossil lineages. As Simpson remarked (ref. 10, p. 173-174) "To students of recent mammals, the association of carnivores and ungulates in a single cohort must appear thoroughly unnatural... Students of early mammals have for some time been increasingly aware that the earliest carnivores and ungulates (*sensu lato*) are more closely similar to each other than either group is to the contemporaneous insectivores and their special allies." Despite these convictions, Simpson did not take great pains to defend various mammalian superordinal categories nor did he make much reference to them in his numerous publications postdating the classification.

The following higher categories were recognized in Simpson's classification (extinct groups are indicated by †):

Class Mammalia
Subclass Prototheria
Order Monotremata (duck-billed platypus, echidna)
Subclass †Allotheria
Order †Multituberculata
Subclass uncertain
Order †Triconodonta
Subclass Theria
Infraclass †Pantotheria
Infraclass Metatheria
Order Marsupialia (opossums, kangaroos and so on)

Infraclass Eutheria
Cohort Ungulata
Order Insectivora (hedgehogs, shrews, moles and so on)
Order Dermoptera (flying lemurs)
Order Primates
Order Chiroptera (bats)
Orders †Tillodontia and †Taeniodonta
Order Edentata (sloths, anteaters, armadillos and so on)
Order Pholidota (pangolins)
Cohort Glires
Order Lagomorpha (rabbits, pikas)
Order Rodentia (rats, mice, porcupines and so on)
Cohort Mutica
Order Cetacea
Cohort Ferungulata
Superorder Ferae
Order Carnivora (including †Creodonta, Fissipeda, Pinnipedia)
Superorder Protungulata
Orders †Condylarthra, †Liptopterna,
†Notoungulata, †Astrapotheria
Order Tubulidentata (aardvarks)
Superorder Paenungulata
Orders †Pantodonta, †Dinocerata, †Pyrotheria
Order Proboscidea (elephants, mastodons and so on)
Order †Embrithopoda
Order Hyracoidea (hyraxes)
Order Sirenia (see cows, manatees)
Superorder Mesaxonia
Order Perissodactyla (horses, rhinos and so on)
Superorder Paraxonia
Order Artiodactyla (even-toed ungulates:
pigs, hippos, camels, deer, cows and so on)

FIG. 1 A phylogenetic tree showing relationships among the major mammalian clades. The solid horizontal bars indicate the age range of the clade on the basis of dated first appearance in the fossil record. Solid lines indicate the branching sequence, although the date of the actual splitting event can only be inferred from the relationships of the clades and their known ages. Dashed lines indicate relatively more ambiguous relationships.



alternative scheme, however, finds almost no support from protein sequence studies^{18,30} that clearly favour the Paenungulata (Fig. 2).

There are also some marked discrepancies between molecular and morphological results. Perhaps most notable among these concerns the Cohort Glires, which includes lagomorphs and rodents. Glires was promoted in early studies⁹, preserved in Simpson's classification¹⁰ and strongly supported in many modern investigations of skull structure, architecture of the ankle joint, fetal membranes and tooth development (for review, see refs 29, 31). Such results starkly contradict immunological comparisons³⁴ and fail to find clear affirmation in protein sequence¹⁸ as well as gene sequence³⁵ studies. Finally, neither molecular nor morphological probes have been successful in resolving major sectors of the mammalian tree.

Morphological and molecular concordance

Yet this somewhat bumpy effort to scan data from complex anatomy to nucleotides has produced some illumination. Both molecular and morphological results seem to converge on the idea that New World edentates (sloths, armadillos and anteaters) represent an early (perhaps even the earliest) branch in the placental mammal tree^{18,22,23,31} (Fig. 1). Although this case is rather tenuous³¹ from an anatomical standpoint, the phylogenetic isolation of edentates is consistent with their long historical isolation in South America²². Protein sequences¹⁸ also suggest a very remote divergence point for edentates. Much greater correspondence is seen in the case of the ungulate branching sequence. For example, results from a cytochrome *b* analysis¹⁹ are compatible with the morphological perspective²⁹ that cetaceans and artiodactyls are segregated from perissodactyls and proboscideans.

But perhaps the most encouraging result to date is the agreement among comprehensive studies of molecules and morphology in certain orders. Such correspondence has been known for some time in primates, where a comparatively large amount of protein sequence data^{17,18} is available. In addition to the cytochrome *b* work¹⁹, mitochondrial (mt) DNA sequences for a sampling of artiodactyls⁵ shows a high compliance with the basic outlines of artiodactyl phylogeny supported by morphological data³⁶. Rapid cladogenesis and early splitting events are best detected with long sequences of highly conserved genes (for example, 18S and 28S ribosomal RNA genes)^{5,37}. Transversions (changes from purine to pyrimidines or vice versa) for these genes seem to have a linear relationship with time for at least 75 million years before present⁵. By contrast, transitions (purine-purine or pyrimidine-pyrimidine changes) in mitochondrial genes are rapid, probably more susceptible to parallelisms, and therefore less reliable as indicators of higher level (superordinal) relationships^{5,37}. Mitochondrial studies on artiodactyls, though confined to a single order of mammals, are

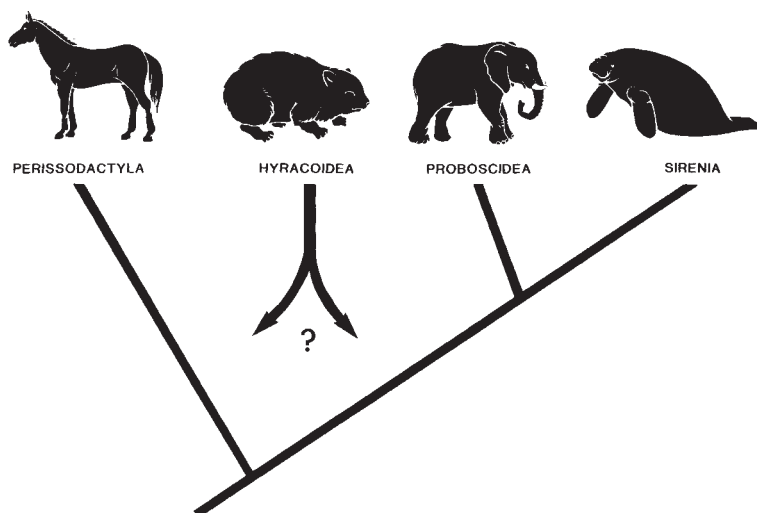
comprehensive enough to suggest that deeper phylogenetic structure might be attained when conservative mitochondrial and nuclear genes are more widely sampled.

Origins of mammalian orders

The current multidisciplinary flavour of mammalian systematics has also taken researchers down long-neglected pathways. The very integrity of certain mammalian orders has been subject to assault. New findings on brain-visual organization have inspired a claim that not all bats (Order Chiroptera) share a unique common ancestor³⁸. The bat diphyly argument (which would probably have caused G. G. Simpson (ref. 10, p. 179) much agitation, has fomented a passionate debate^{39,40}, conducted mainly on the grounds of conflicting anatomical evidence. Recent molecular and some new morphological data are clearly more consistent with the traditional view of a single origin for bats (Fig. 3).

Another unexpected attack has been made on traditional concepts of rodent origins. Central to theories concerning rodent phylogeny¹² is the notion that the separation of the hystricognaths from other rodents represents a very early divergence

FIG. 2 The debate over hyracoid relationships has been spawned by a curious mismatch of anatomical evidence. Although the Paenungulata (Hyracoidea, Sirenia and Proboscidea) is affirmed by specializations involving the serial arrangement of the carpal elements and the reduction of certain basicranial bones and other features²⁹, at least some living perissodactyls and hyracoids share notable and rather bizarre specializations including an expanded eustachian sac in the middle ear region³². Nonetheless, molecular results, primarily amino-acid sequence data from lens α -crystallin and other proteins^{18,30}, clearly support the paenungulate grouping, as does a palaeontological accounting of early fossil horses⁴⁸. New features of ear morphology in fossil proboscideans⁸ have significant bearing on the origin of this group and their relationships with sirenians, hyraxes and other ungulates.



event. Recently, this distinction for hystricognaths has been carried to a new extreme. It has been proposed⁴¹ that the removal of the hystricognath *Cavia porcellus* (the guinea pig) from the Rodentia resolves some of the paradoxical qualities of gene evolution in this species, including rampant parallelisms with molecular changes observed in birds and drastically accelerated rates of gene evolution. The idea is provocative, but it minimizes the impressive morphological evidence for the monophyly of Rodentia inclusive of hystricognaths^{9,10,42}. A recent analysis of 12S rRNA genes⁴³ showed that transversions applied to an unrooted network of four eutherian mammal taxa support rodent monophyly. In the same study⁴³, a network rooted with a non-mammal outgroup (chicken) disassembled Rodentia. But this solution required only one mutation less than the tree opting for rodent monophyly. The higher statistical support for rodent monophyly in the unrooted analysis suggests that a bird taxon is too remotely related to be a reference outgroup. An alternative (perhaps a marsupial or an edentate) would provide a better test. In any case, the molecular sampling is as yet too sketchy to resolve the issue.

New fossil evidence

Given the energies invested in molecular research, it might be assumed that the repository of more traditional (that is, anatomical and palaeontological) evidence has been depleted. This is not the case. Recent and extraordinary discoveries of fossils continue to shake the tree. Prominent among these is the disclosure that a group of early primate-like taxa, once known primarily from dentitions, have skeletons that show bizarre adaptations similar to the gliding dermopterans⁶. The discovery has reopened the whole question of primate affinities with dermopterans and other putatively related orders. For some time⁹, the monophyly of a Superordinal category the Archonta has been recognized to include the primates, dermopterans, tree shrews (Order Scandentia) and the bats. One concept favours the close affinity between dermopterans and bats on one hand and tree shrews and primates on the other^{29,30,31}. Nucleotide sequences of cytochrome oxidase (COII) genes⁴⁴ in a sampling of archontans suggests that tree shrews are no more closely related to primates than are flying lemurs. Interestingly, the COII study weakens the case for the Archonta by isolating the monophyletic Chiroptera from the other archontan groups.

Another remarkable advance on the palaeontological front is the discovery of evidence in Eocene whales⁷ that elucidates the long-suspected connection between cetaceans and early artiodactyl relatives. Vestigial hindlimbs in the Eocene *Basilosaurus* distally show a paraxonic arrangement⁷, a condition where digits symmetrically extend about a plane between digits III and IV (digit II is essentially lacking in *Basilosaurus*).

This paraxonic arrangement bears striking resemblance to that in an archaic group of ungulates, the mesonychid condylarths, as well as the even-toed (paraxonic) artiodactyls^{7,45}. Mesonychid condylarths are, moreover, very like primitive whales (archaeocetes) in skull and dental structure. The picture resolves to a branching sequence wherein whales and artiodactyls are closely related by virtue of their close affinities with different groups of mesonychids. Immunodiffusion, protein sequence⁴⁶ and cytochrome *b* sequence¹⁹ results suggest an even more intimate derivation of cetaceans somewhere within

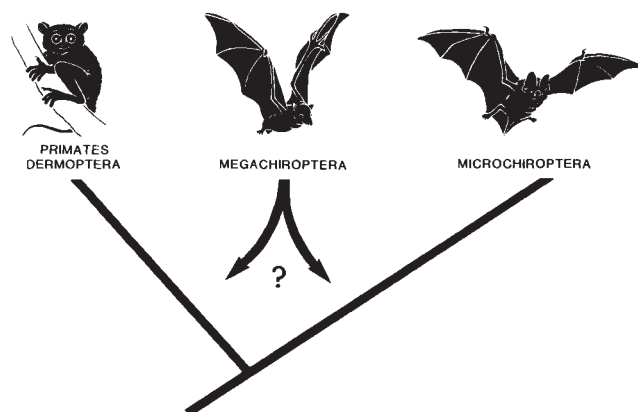


FIG. 3 Contrary to the standard view of bat monophyly, some workers argue that the Suborder Megachiroptera (which includes the large Old World fruit bats) is more closely related, by virtue of specialized visual neural pathways in the brain, to primates (and possibly also to flying lemurs and tree shrews) than to the echolocating Microchiroptera³⁸. Under this view, myriad similarities in wing structure and the unique capabilities for powered flight evolved independently in the two bat groups, whereas the brain-visual traits, being less obviously adaptive, are clearly indicative of a true genealogical relationship³⁹. Opponents stress that this argument has been tied to a naive assumption linking the obvious adaptation of a feature with a high likelihood that it is not good evidence for monophyly⁴⁰. Such an assumption, they argue, would effectively eliminate many essential characters (including the notochord in chordates, or mammary glands in mammals) from use in biological classifications. From the molecular side, restriction map analysis of the ribosomal DNA gene complex²⁰ failed to clearly indicate either bat monophyly or diphyly. But amino-acid sequences relating to three globin genes⁸⁶, and DNA sequence changes in 12S rRNA^{87,88}, cytochrome oxidase subunit I (COI)⁸⁷ and cytochrome oxidase subunit II (COII)⁴⁴ all strongly support a single origin for bats. New morphological evidence on the intricate and unique nature of innervation of appendicular and wing musculature⁸⁹ further strengthens the case for chiropteran monophyly. Bats are generally associated with primates, flying lemurs and tree shrews in the Superorder Archonta^{9,29,90}. Finally, recent neurological work⁹¹ suggests that brain visual traits may be misleading evidence for bat diphyly.

Artiodactyla. Nonetheless, in a general sense, the cetacean-artiodactyl association so well illuminated by the fossil record is supported by the new molecular data. The impact of palaeontology here and in other cases should not be underestimated. Fossils will continue to reveal crucial branching points in the deep structure of mammalian phylogeny, patterns that might never be retrieved from the molecular and anatomical structure of extant taxa alone^{47,48}.

The insectivore problem

The importance of the palaeontological database is also demonstrated by the reorganization and clarification of the Insectivora, a group central to the question of higher relationships among placental mammals. As originally conceived, Insectivora comprised hedgehogs, moles, shrews, tenrecs, solenodons, golden moles and a diverse suite of fossils in the Suborder Lipotyphla along with tree shrews (Scandentia) and elephant shrews (Macroscelidea) in the suborder Menotyphla. For some time, however, Menotyphla has been rendered obsolescent⁹ by systematic fission. As noted above, tree shrews have been drawn in with primates, bats and flying lemurs^{9,29}. Elephant shrews have been moved toward the Glires complex²⁹, but recent molecular and morphological studies spread them over the mammalian map, providing contradictory evidence for affinities with various archontans⁴⁹ or primitive ungulates⁵⁰ or for a remote branching position at the base of the eutherian tree⁵¹.

This distillation notwithstanding, Insectivora still presented many problems, largely created by the association of lipotyphlans with palaeoryctids, leptictids and other fossil taxa of primitive skeletal structure. As the fossil evidence accumulated, Insectivora simply became a receptacle for any taxon that defied reasonable allocation elsewhere⁵². A reassessment of well preserved fossils^{53–56} has etched a clearer distinction between archaic insectivorans and those of a more modern stamp. There is now some case for the monophyly of a more restricted Lipotyphla^{53,56}. The fossil leptictids are clearly excluded from this assemblage, although their skull structure suggests they may be the nearest relatives of the lipotyphlans⁵⁶. Cretaceous palaeoryctids are of extremely primitive morphology⁵⁵ and probably diverged much earlier in the eutherian branching sequence⁵⁶ (Fig. 1).

Carnivores and the origins of pinnipeds

Problems of insectivore phylogeny readily merge with questions concerning carnivore origins. The sharp, high-crowned teeth of early fossil carnivores evoke comparisons with archaic insectivorans, particularly palaeoryctids⁵⁶. Unfortunately, the relations of carnivores with other extant orders remains enigmatic. This uncertainty also applies to Simpson's proposal for a carnivore-ungulate connection in the Cohort Ferungulata (Box 1). A recent study of gene sequences³⁵ in a few mammalian species suggests a closer affinity between carnivores and artiodactyls than between the former and either primates or rodents. Nonetheless, taxonomic coverage for gene sequence comparisons relevant to this issue must be greatly expanded.

Within Carnivora, the picture is more satisfactorily resolved both from morphological^{12,57} and from molecular⁵⁸ perspectives, and it bears on an important problem in mammalian phylogeny. Although there is resounding consensus that pinnipeds (seals, sea lions and walruses) are closely related to terrestrial carnivores, there is much controversy over the nature of this linkage. Throughout the twentieth century, pinnipeds were most consistently viewed as monophyletic⁵⁹, but a diphyletic origin for pinnipeds has also been proposed^{60–62}. The diphyletic argument calls for the alliance of odobenids (walruses) and otariids (sea lions) within an arctoid clade somewhere near ursids (bears) and a separate connection between the phocids (seals) and mustelids (weasels, skunks, and kin). But recent morphological work⁶³ revives the monophyletic argument with an added refinement suggesting a close odobenid-phocid affinity.

Molecular studies emphatically support a case for pinniped monophyly^{63–66}.

Marsupials and monotremes

The problems noted so far apply to the diverse orders assigned to the Infraorder Eutheria, the placental mammals. The other major radiation of living mammals, represented by the Australian and New World marsupials, has also attracted much attention, although developments on the molecular front are less dramatic. Recent proposals^{67–69} favour basic divisions that roughly parallel the pattern of biogeographic separation between Australian and New World marsupials. Intriguingly, some of these classifications^{67,68} converge on the notion that the New World microbiotheres (whose sole living member is *Dromiciops*, the Monitos del Monte) represents the first twig of the branch leading to the Australian radiation. Indeed, the discovery of fossil marsupials in Antarctica⁷⁰ conforms with the long-standing view that New World and Australian marsupials diverged from a Mesozoic group distributed broadly over then connected landmasses of South America, Antarctica and Australia.

So far, the molecular input on questions of marsupial relationships primarily concern immunological^{71,72} and DNA-DNA hybridization^{73–75} studies. Some immunological comparisons allegedly support the close affinity between Australian marsupials and microbiotheres (see comment, p. 457 in ref. 67), but this is not clearly indicated by DNA-DNA hybridization results⁷⁵. Protein and gene sequencing studies^{51,58} merely corroborate the expected aggregation of a few representative marsupials (for example, opossums and kangaroos). The nascent area of gene sequence studies in marsupials includes findings⁷⁶ on 12S rRNA and cytochrome *b* which link the extinct *Thylacinus* (the marsupial wolf) with the Australian *Dasyurus* (tiger cat) and *Sarcophilus* (Tasmanian devil), an association compatible with some current classifications⁶⁷.

Until recently, the fossil record of the presumably oldest but least diverse of the major mammalian clades, the monotremes, was limited to scattered and partial remains in the Late Cenozoic of Australia. That the fossil record still offers many surprises is fully demonstrated by findings in Australia of an exquisite Miocene platypus skull⁷⁷ and a Cretaceous jaw and dentition also claimed to represent an early platypus⁷⁸. These specimens not only provide new data on the evolution of monotreme characters, but also indicate a closer relationship than was traditionally recognized⁷⁸ between monotremes and the other extant mammalian clades relative to various Mesozoic mammals.

Search for congruence

The above cases show a mixed picture of an attack, with a diverse arsenal, on a very tough problem. The goal here is, however, important. A better map of mammalian history provides a critical framework for wrestling with the problems of genic and taxic rates of change, biogeographic deployment and the connection between phylogeny and character and gene transformation³¹. It is, of course, difficult to measure progress in fashioning this framework. For one thing, agreement among different data sets does not guarantee that we are on the right track. Moreover, the failure to achieve congruence may simply mean that not all approaches are equally effective in revealing phylogeny. Critics^{23,53} note that an abiding fascination with tooth structure seems responsible for some very confusing and inefficient descriptions of higher mammal relationships. Dental traits seem prone to rapid and parallel change. Likewise, certain genes and proteins seem ineffective in retrieving ancient splitting events⁷⁹ because of their tendency for multiple changes at a single locus after the split¹⁹. Indeed, a recent analysis²⁸ suggests that amino-acid replacements in certain proteins produced trees that were in fact less efficient in describing the variation than trees produced from a random mix of characters and taxa.

These shortcomings, and the conflicting results they produce,

make one look more carefully at the nature of the basic evidence. Nevertheless, in the absence of knowledge of the true phylogeny, a measure of congruence among data sets must be heeded. This is why congruent patterns produced from molecular data, morphological traits of various systems, and the fossil record are greeted with some feeling of comfort³⁷. If a signal for phylogeny is there, it should be seen from several different directions.

Prospects

At present, the study of higher mammalian phylogeny is so carried by its own momentum that it seems presumptuous to consider where and how new insights will arrive. There is an obvious, auspicious direction to the study of gene sequences, with merely the threshold attained for survey of the more conserved, and possibly more illuminating, genes⁸⁰. Another promising route here concerns the experimental disclosure of genic influence on mammalian development and morphology⁸¹, as a basis for further insights on character transformation in phylogeny. Likewise, the fossil record of mammals seems capable of unabated offerings of new evidence. Where do the sort of 'middle of the road' studies of morphology in living taxa fit? There is a tendency to observe that such approaches, despite their long cultivation, have not yielded their share of productive

results⁸². But if this is true, why is there startling new phylogenetic information on brain-visual systems³⁸, fetal development⁸³ and minutiae of the middle ear²⁹? In reality, the traditional study of morphology has little to do with the new cladistic methods for seeking homology, coding data, and searching for trees that today bring together morphological and molecular research. One can recast morphological studies, as well as molecular systematics⁸², as a new age of exploration.

Will all this interaction eventually produce a precise and elegant picture of higher mammal phylogeny? There is no reason to expect that all the basic lines among, for instance, the placental mammal orders will be teased apart. And there may be some truth in Simpson's remarks^{31,84} that the great burst of radiating mammalian orders more than 65 million years ago will not completely yield to these probes. Simpson neither encouraged further research on the subject nor did he seem very enchanted⁸⁵ with one of the methods (namely, cladistics) now adopted to carry it out. Yet enough insight and collaboration has come from this venture, that its momentum seems justified. It is hard to imagine that even Simpson himself wouldn't have agreed. □

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- Hennig, W. *Phylogenetic Systematics* (Univ. Illinois Press, Urbana, 1966).
- Nelson, G. & Platnick, N. I. *Systematics and Biogeography: Cladistics and Vicariance* (Columbia Univ. Press, New York, 1981).
- Hillis, D. M. & Dixon, M. T. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 355–367 (Elsevier, Amsterdam, 1989).
- Miyamoto, M. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7627–7631 (1988).
- Miyamoto, M. M. & Boyle, S. M. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 437–450 (Elsevier, Amsterdam, 1989).
- Beard, K. C. *Nature* **345**, 340–341 (1990).
- Gingerich, P. D., Smith, B. H. & Simons, E. L., *Science* **249**, 154–157 (1990).
- Court, N. & Jaeger, J. J. C. *Revue. Séanc. Acad. Sci., Paris* **3129**, 559–565 (1991).
- Gregory, W. K. *Bull. Am. Mus. nat. Hist.* **27**, 1–542 (1910).
- Simpson, G. G. *Bull. Am. Mus. nat. Hist.* **85**, 1–350 (1945).
- Simpson, G. G. *Tempo and Mode in Evolution* (Columbia Univ. Press, New York, 1944).
- Carroll, R. L. *Vertebrate Paleontology and Evolution* (Freeman, New York, 1988).
- Avery, O. T., MacLeod, C. M. & McCarthy, M. J. *exp. Med.* **79**, 137–158 (1944).
- Zuckerkandl, E. & Pauling, L. in *Horizons in Biochemistry* (eds Kash, M. & Pullman, B.) 189–225 (Academic, New York, 1962).
- Salich, V. M. *Syst. Zool.* **18**, 286–295 (1969).
- Goodman, M. & Moore, G. W. *Syst. Zool.* **20**, 19–62 (1971).
- Goodman, M., Moore, G. W. & Matsuda, G. *Nature* **253**, 603–608 (1975).
- Miyamoto, M. M. & Goodman, M. *Syst. Zool.* **35**, 230–240 (1986).
- Irwin, D., Kocher, T. D. & Wilson, A. C. *J. molec. Evol.* **32**, 128–144 (1991).
- Baker, R. J., Honeycutt, R. L. & Van Den Bussche, R. A. *Bull. Am. Mus. nat. Hist.* **206**, 42–53 (1991).
- White, T. J., Arnheim, N. & Erlich, H. A. *Trends Genet.* **5**, 185–189 (1989).
- McKenna, M. C. in *Phylogeny of the Primates* (eds Luckett, W. P. & Szalay, F. S.) 21–46 (Plenum, New York and London, 1975).
- Novacek, M. J. in *Macromolecular Sequences in Systematic and Evolutionary Biology* (ed. Goodman, M.) 3–41 (Plenum, New York, 1982).
- Swofford, D. L. *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.0* (Illinois Natural History Survey, Champaign, Illinois, 1989).
- Farris, J. S. *HEWING 86, Version 1.5* (Distributed by the author: 41 Admiral Street, Port Jefferson Station, New York, 1988).
- Waterman, M. S. *Bull. math. Biol.* **46**, 473 (1984).
- Wheeler, W. C. & Honeycutt, R. L. *Molec. Biol. Evol.* **5**, 90–96 (1988).
- Faith, D. P. & Cranston, P. S. *Cladistics* **7**, 1–28 (1991).
- Novacek, M. J., Wyss, A. R. & McKenna, M. C. in *The Phylogeny and Classification of the Tetrapods* Vol. 2 (ed. Benton, M. J.) 31–71 (Clarendon, Oxford, 1988).
- McKenna, M. C. in *Molecules and Morphology in Evolution: Conflict or Compromise?* (ed. Patterson, C.) 55–93 (Cambridge Univ. Press, 1987).
- Novacek, M. J. in *Current Mammalogy* Vol. 2 (ed. Genoways, H. H.) 507–543 (Plenum, New York, 1990).
- Prothero, D. R., Manning, E. M. & Fischer, M. in *The Phylogeny and Classification of the Tetrapods* Vol. 2 (ed. Benton, M. J.) 201–234 (Clarendon, Oxford, 1988).
- Owen, R. *J. geol. Soc. Lond.* **4**, 103–141 (1848).
- Salich, V. M. in *Evolutionary Relationships among Rodents* (eds Luckett, W. P. & Hartenberger, J.-L.) 423–452 (Plenum, New York and London, 1985).
- Li, W.-H., Guoy, M., Sharp, P. M., O'Huigin, C. & Yang, Y.-Y. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6703–6707 (1990).
- Janis, C. M. & Scott, K. M. *Am. Mus. Novit.* **2893**, 1–85 (1987).
- Kraus, F. & Miyamoto, M. M. *Syst. Zool.* **40**, 117–130 (1991).
- Pettigrew, J. D. *Science* **231**, 1304–1306 (1986).
- Pettigrew, J. D. *Syst. Zool.* **40**, 199–216 (1991).
- Baker, R. J., Novacek, M. J. & Simons, N. B. *Syst. Zool.* **40**, 216–231 (1991).
- Graur, D., Hide, W. A. & Li, W.-H. *Nature* **351**, 649–652 (1991).
- Luckett, W. P. & Hartenberger, J.-L. in *Evolutionary Relationships among Rodents* (eds Luckett, W. P. & Hartenberger, J.-L.) 685–712 (Plenum, New York and London, 1985).
- Allard, M. W., Miyamoto, M. M. & Honeycutt, R. L. *Nature* **353**, 610–611 (1991).
- Adkins, R. M. & Honeycutt, R. L. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10317–10321 (1991).
- Wyss, A. R. *Nature* **347**, 428–429 (1990).
- Goodman, M., Czelusniak, J. & Beebe, J. E. *Cladistics* **1**, 171–185 (1985).
- Gauthier, J., Kluge, A. G. & Rowe, T. *Cladistics* **4**, 105–209 (1988).
- Novacek, M. J. *Syst. Biol.* (in the press).
- Woodall, P. F. *J. submicrosc. Cytol. Pathol.* **23**, 47–58 (1991).
- Simmons, E. L., Holroyd, P. A. & Bown, T. M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9734–9737 (1991).
- McKenna, M. C. *Acta zool. fenn.* **191** (in the press).
- Butler, P. M. in *Studies in Vertebrate Evolution* (eds Joysey, K. A. & Kemp, T. S.) 253–265 (Winchester, New York, 1972).
- McDowell, S. B. *Bull. Am. Mus. nat. Hist.* **115**, 113–214 (1958).
- Butler, P. M. *Proc. zool. Soc. Lond.* **118**, 453–481 (1956).
- Kielan-Jaworowska, Z. *Palaeont. pol.* **33**, 5–13 (1975).
- Novacek, M. J. *Bull. Am. Mus. nat. Hist.* **183**, 1–111 (1986).
- Flynn, J. J., Neff, N. A. & Tedford, R. H. in *The Phylogeny and Classification of the Tetrapods* Vol. 2 (ed. Benton, M. J.) 73–115 (Clarendon, Oxford, 1988).
- Czelusniak, J., Goodman, M., Moncrief, N. D. & Kehoe, S. M. *Meth. Enzym.* **183**, 601–615 (1990).
- Flower, W. H. *Proc. zool. Soc. Lond.* **1869**, 4–37 (1869).
- Mivart, St. G. *Proc. zool. Soc. Lond.* **1885**, 484–500 (1885).
- McLaren, I. A. *Syst. Zool.* **9**, 18–28 (1960).
- Tedford, R. H. *Syst. Zool.* **25**, 363–374 (1976).
- Wyss, A. R. *Am. Mus. nat. Hist. Novit.* **2871**, 1–13 (1987).
- Arnason, U. *Hereditas* **76**, 179–226 (1974).
- Goodman, M., Romero-Herrera, A. E., Dene, H., Czelusniak, J. & Tashian, R. E. in *Macromolecular Sequences in Systematics and Evolution* (ed. Goodman, M.) 115–191 (Plenum, New York, 1982).
- Romero-Herrera, A. E., Lehmann, H., Joysey, K. A. & Friday, A. E. *Phil. Trans. R. Soc. B* **283**, 61–163.
- Marshall, L. G., Case, J. A. & Woodburne, M. O. in *Current Mammalogy* Vol. 2 (ed. Genoways, H. H.) 433–505 (Plenum, New York, 1990).
- Applin, K. & Archer, M. in *Possums and Opossums: Studies in Evolution* (ed. Archer, M.) xv–lxxii (R. zool. Soc. NSW, Sydney, 1987).
- Clemens, W. A., Richardson, B. J. & Baverstock, P. R. in *Fauna of Australia* Vol. 1B (eds Walton, D. W. & Richardson, B. J.) 527–548 (Australian Government Publishing Service, Canberra, 1989).
- Woodburne, M. O. & Zinsmeister, W. J. *J. Paleont.* **58**, 913–948 (1984).
- Kirsch, J. A. W. *Austr. J. Zool.* **52** (suppl.), 1–152 (1977).
- Baverstock, P. R., Birrell, J. & Krieg, M. in *Possums and Opossums: Studies in Evolution* (ed. Archer, M.) 229–234 (R. zool. Soc. NSW, Sydney, 1987).
- Kirsch, J. A. W., Krajewski, C., Springer, M. S. & Archer, M. *Aust. J. Zool.* **38**, 673–696 (1990).
- Springer, M. S., Kirsch, J. A. W., Applin, K. & Flannery, T. *J. molec. Evol.* **30**, 298–311 (1990).
- Westerman, M. & Edwards, D. *Aust. J. Zool.* **39**, 123–130 (1991).
- Thomas, R. H., Schaffner, W., Wilson, A. C., Pääbo, S. *Nature* **340**, 465–467 (1989).
- Archer, M., Hand, S. & Godthelp, H. *Uncovering Australia's Dreamtime* (Surrey Beatty, Chipping Norton, New South Wales, 1986).
- Archer, M., Flannery, T. F., Ritchie, A. & Molnar, R. *Nature* **318**, 363–366 (1985).
- Wyss, A. R., Novacek, M. J. & McKenna, M. C. *Molec. Biol. Evol.* **4**, 99–116 (1987).
- Holmes, E. C. *J. molec. Evol.* **33**, 209–215 (1991).
- Kessler, M. & Gruss, P. *Cell* **67**, 89–104 (1991).
- Goodman, M. in *The Hierarchy of Life* (eds Fernholm, B., Bremer, K. & Jönvall, H.) 43–61 (Elsevier, Amsterdam, 1989).
- Luckett, W. P. in *Evolutionary Relationships among Rodents* (eds Luckett, W. P. & Hartenberger, J.-L.) 227–276 (Plenum, New York and London, 1985).
- Simpson, G. G. *Proc. Am. Phil. Soc.* **122**, 318–328 (1978).
- Simpson, G. G. in *Phylogeny of the Primates* (eds Luckett, W. P. & Szalay, F. S.) 3–19 (Plenum, New York and London, 1975).
- Czelusniak, J. et al. in *Current Mammalogy* Vol. 2 (ed. Genoways, H. H.) 545–572 (Plenum, New York, 1990).
- Mindell, D. P., Dick, C. W. & Baker, R. J. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10322–10326 (1991).
- Ammerman, L. & Hillis, D. M. *Am. Zool.* **30**, 50 (1990).
- Thewissen, J. G. M. & Babcock, S. K. *Science* **251**, 934–936 (1991).
- Szalay, F. S. in *Major Patterns in Vertebrate Evolution* (eds Hecht, M. K., Goody, P. C. & Hecht, B. M.) 315–374 (Plenum, New York, 1977).
- Thiele, A., Vogelsang, M., Hoffmann, K.-P. *J. comp. Neur.* **314**, 671–683 (1991).

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Crystal structure of four-stranded *Oxytricha* telomeric DNA

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The sequence d(GGGGTTTTGGGG) from the 3' overhang of the *Oxytricha* telomere has been crystallized and its three-dimensional structure solved to 2.5 Å resolution. The oligonucleotide forms hairpins, two of which join to make a four-stranded helical structure with the loops containing four thymine residues at either end. The guanine residues are held together by cyclic hydrogen bonding and an ion is located in the centre. The four guanine residues in each segment have a glycosyl conformation that alternates between *anti* and *syn*. There are two four-stranded molecules in the asymmetric unit showing that the structure has some intrinsic flexibility.

TELOMERES are located at the ends of chromosomes and contain DNA with specialized sequences. They have an essential role in maintaining the stability and integrity of chromosomes (reviewed in ref. 1). The sequences are made up of short nucleotide repeats and characteristically have a G-rich and a C-rich strand. Sequenced telomere ends have two repeats of the G-rich strand which overhang at the 3' end^{2,3} and they have unusual properties⁴⁻⁷. For example, in the macronucleus of the ciliated protozoan *Oxytricha*, telomere ends form cohering structures^{4,5,8}. Several models have been proposed for the three-dimensional structure of the G-rich strand of telomers⁹⁻¹¹. These models generally involve four guanine bases that are hydrogen-bonded to each other in a cyclic fashion (G-quartet). The origin of these models stems from experiments over 30 years ago in which the structure of fibres of polyinosinic acid was studied¹². The fibres had a diffraction pattern consistent with the formation of three-stranded or four-stranded molecules in which the central bases form cyclic hydrogen bonds. Polyinosinic acid was later shown to be four-stranded^{13,14}. A similar system of cyclic hydrogen-bonding of four guanine bases was used to interpret the stable gels formed by monomeric guanylic acid¹⁵, as well as polyguanylic acid^{13,14}. When guanine is used, eight hydrogen bonds are thought to stabilize the structure at each level of the helix. Structures of this type have also been proposed to be important in meiosis in regions of the chromosomes where there are stretches of G residues with four guanine strands parallel to each other¹⁶.

Experiments dealing with the G-rich telomere sequences have led to the proposals that they can form four-stranded structures in which the two repeats of the 3'-terminal G strand are formed into hairpin-like structures which combine to form quartets of bases⁹⁻¹¹. These models were based on an analysis of gel electrophoretic patterns, chemical modifications and ultraviolet-induced crosslinks. But until now there have been no single crystal X-ray analyses of any structure containing four guanine bases hydrogen-bonded in a cyclic fashion.

The 3'-terminal overhang of *Oxytricha* has the sequence

d(T₄G₄)₂ (ref. 4). We have synthesized d(G₄T₄G₄), crystallized it and solved its three-dimensional structure. This telomeric sequence forms a complex containing two d(G₄T₄G₄) molecules held together by cyclic hydrogen bonding of four guanines. The two thymine tracts form loops found at opposite ends of the molecule, and a potassium ion is located at the centre of the quartets of guanine bases. Unlike the proposed models, however, the stretches containing guanine residues alternate in *syn* and *anti* conformation in agreement with nuclear magnetic resonance (NMR) experiments¹⁷⁻¹⁹.

Crystallization and structure determination

Many G-rich telomeric sequences were synthesized (Applied Biosystems Model 380B), but the most promising crystals were obtained with d(G₄T₄G₄) from *Oxytricha*. The space group was *P*2₁2₁2₁, *a* = 27.73 Å, *b* = 49.57 Å, *c* = 97.27 Å. There is room for four d(G₄T₄G₄) strands in the asymmetric unit. The structure was solved by molecular replacement and a Patterson correlation refinement²⁰ using four-guanine quartet models with alternate chains running in various directions both right- and left-handed. The right-handed coordinates were mostly derived from those of Zimmerman²¹ with modifications in that alternate strands had either all *anti* or all *syn* conformation of the guanine residues. The right-handed conformation produced two significant solutions. These two positions were related by a pseudo 2-fold axis identical to the solution of the self-rotation search. After rigid body refinement of this partial model, difference maps were constructed which made it possible to trace the loops of the four thymine residues which were found on opposite ends of the G-quadruplex. Molecular replacement and simulated annealing refinement were done with the program X-PLOR²² and graphics with FRODO²³. Omit maps were made to determine chain polarity and glycosyl conformation. After restrained individual temperature factors were refined, a number of ions or solvent molecules were located. The final structure contained, in addition to the four strands of d(G₄T₄G₄), 160 ions or solvent molecules, including spermines. Crystallographic data and refinement statistics are presented in Table 1 and full details will be published elsewhere. The coordinates have been deposited in the Brookhaven Protein Data Bank.

Views of the telomeres

Each strand folds back on itself forming an intramolecular hairpin stabilized by G · G base pairs⁶. Two of these hairpins associate together in an *anti* parallel manner to form a stack of four guanine quartets, with thymine tract loops at each end of the molecule. The loops join the two G tracts on the same side of the quartet rather than across the diagonal. The structure has two wide and two narrow grooves. The latter are formed by the two 5' ends of the molecule or the two 3' ends (Fig. 1). The numbering scheme that was used is shown in Fig. 2. In numbering the residues, we have taken the first guanine residue at the 5' position to be the one closest to the pseudo 2-fold axis. There are two molecules A and B and the numbering system is shown in Fig. 2. Molecules A and B are joined together by a symmetric pair of hydrogen bonds from the 3' OH of G12 and G42 to phosphate oxygens of G42 and G12, respectively. The proximal

TABLE 1 Refinement summary

Resolution (Å)		10.0–3.91	3.13	2.75	2.50	2.30	Total
Possible		1,303	1,228	1,209	1,203	1,379	6,322
Measured	(all†)	1,177	1,163	963	905	755	4,963
	(1 σ ‡)	1,139	1,061	713	621	451	3,985
	(2 σ)	997	780	332	244	118	2,471
<i>R</i> -factor* (%)	(1 σ)	26.3	21.2	23.2	23.9	24.7	23.8
	(2 σ)	23.0	16.5	17.3	17.6	19.3	19.5

Owing to the pseudo 2-fold axis parallel to the *y* axis and the resultant pseudo centering, a large proportion of the reflections are weak. We have not used non-crystallographic symmetry constraints between molecules A and B in the refinement. R.m.s. deviation of bond distance: 0.016 Å, bond angle: 3.99°.

* *R*-factor = $\sum |F_o - F_c| / \sum |F_o|$.

† All: above zero data.

‡ σ : based on I.

thymine loops of molecules A and B are close to each other and several ions are found interposed near the pseudo 2-fold axis. The helical axes of molecules A and B are oriented 125° from each other. A and B have very similar structures and the best molecular fitting between them gives an r.m.s. deviation of 1.48 Å. Comparison of the G-quartets alone has an r.m.s. deviation of 1.29 Å. Thus the largest differences between the two molecules was found in the organization of the thymine loops.

The distribution of electron density and the position of guanine residues in the first and second planes of molecule B is shown in Fig. 3a. The cyclically bonded guanine residues do not form a completely square array, but are slightly distorted. This is less so for the second and third planes, which may be associated with the presence of an ion located between them as discussed below. Because of the distortions from a square array

in some planes, several hydrogen bonds are three-centred or bifurcated.

The core of the molecule consists of four stacked G quartets with a mean spacing of 3.5 Å. Figure 3b shows a projection of molecule A viewed in its electron density showing the four planes of the quadruplex as well as three of the four thymine residues in the loop. The disposition of sugar phosphate chains in electron density are shown in Fig. 4. Rounded segments of electron density found between the chains are solvent molecules or ions. Inspection of the guanine residues attached to the chain shows the manner in which they alternate in *syn* and *anti* conformations. Figure 2 lists the conformation of the glycosyl bonds, either *anti* (A) or *syn* (S). Glycosyl torsion angles are also listed for the guanine residues in both chains. The mean difference in the glycosyl angle of corresponding bases in molecules A and B is 23°.

FIG. 1 Stereo view of the *Oxytricha* telomere structure looking down the pseudo 2-fold axis parallel to the *y*-axis. The upper molecule is designated A and the lower B. It can be seen that the molecule has grooves that are wide and grooves that are narrow between the sugar phosphate backbones. The narrow grooves shown here contain the two 3' ends of the molecules. Crystals were grown by vapour diffusion using the hanging drop technique from a solution that contained 1 mM oligonucleotide, 10 mM MgCl₂, 6 mM spermine, 40 mM KCl, 20 mM potassium cacodylate buffer (pH 7.0), 5% 2-methylid-2,4-pentane-diol (MPD) equilibrated with a reservoir of 40% MPD. three-dimensional X-ray diffraction data were collected to 2.3 Å resolution using the multiwire Xuong-Hamlin area detector at the UCSD Research Resource.

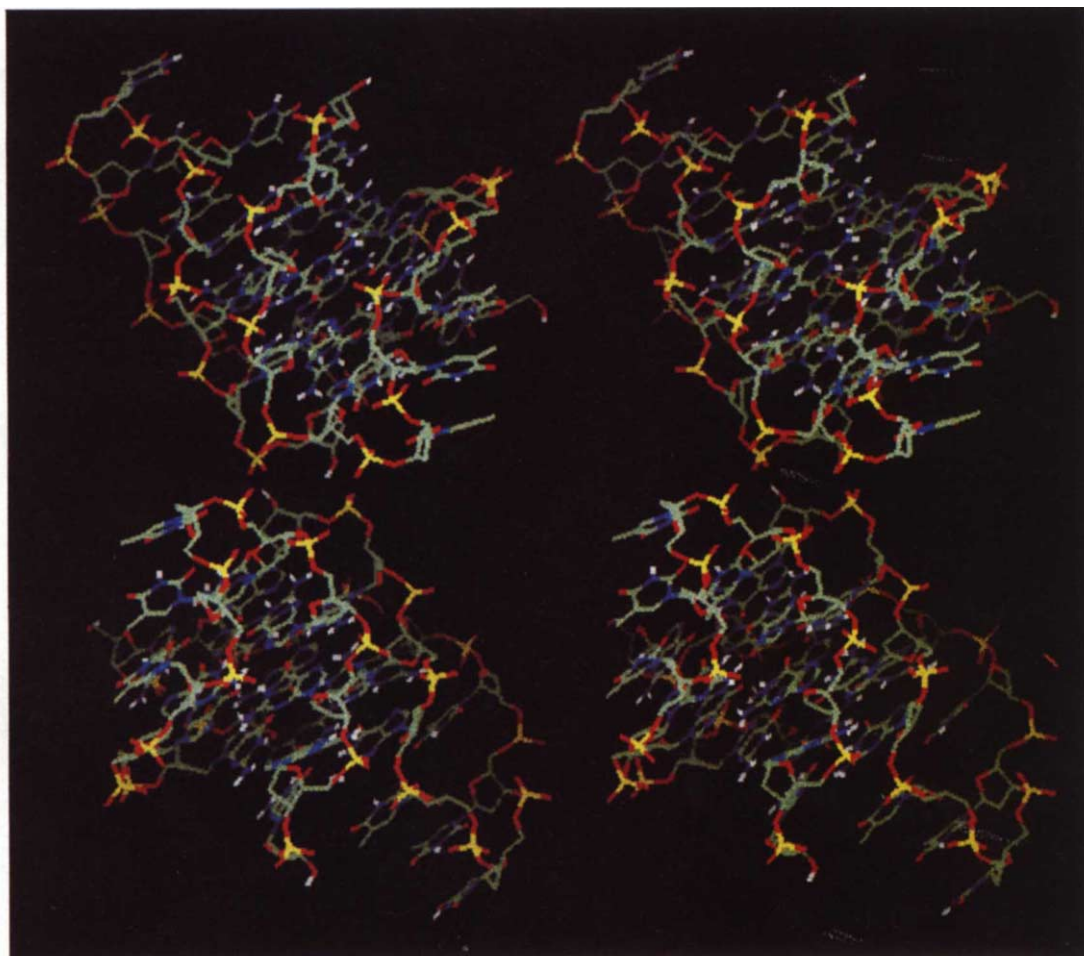


FIG. 2 The numbering system of the A molecule in the *Oxytricha* telomere structure. G1 is the 5' end of one strand closest to the pseudo 2-fold axis. G1–G12 is one strand, G13–G24 is the other. Next to each guanine residue, S indicates *syn* and A indicates *anti* conformations around the glycosyl bond. Molecule B (not shown) is numbered G30–G42 in one strand, G43–G54 in the other. The two numbers next to the guanine symbols show the glycosyl torsion angles for the base. The number in parenthesis refers to the B molecule. The narrow grooves have either the 3' ends or the 5' ends of both strands. The asterisk next to G1 at the 5' end indicates that its sugar is in a high *syn* conformation because of hydrogen-bonding.

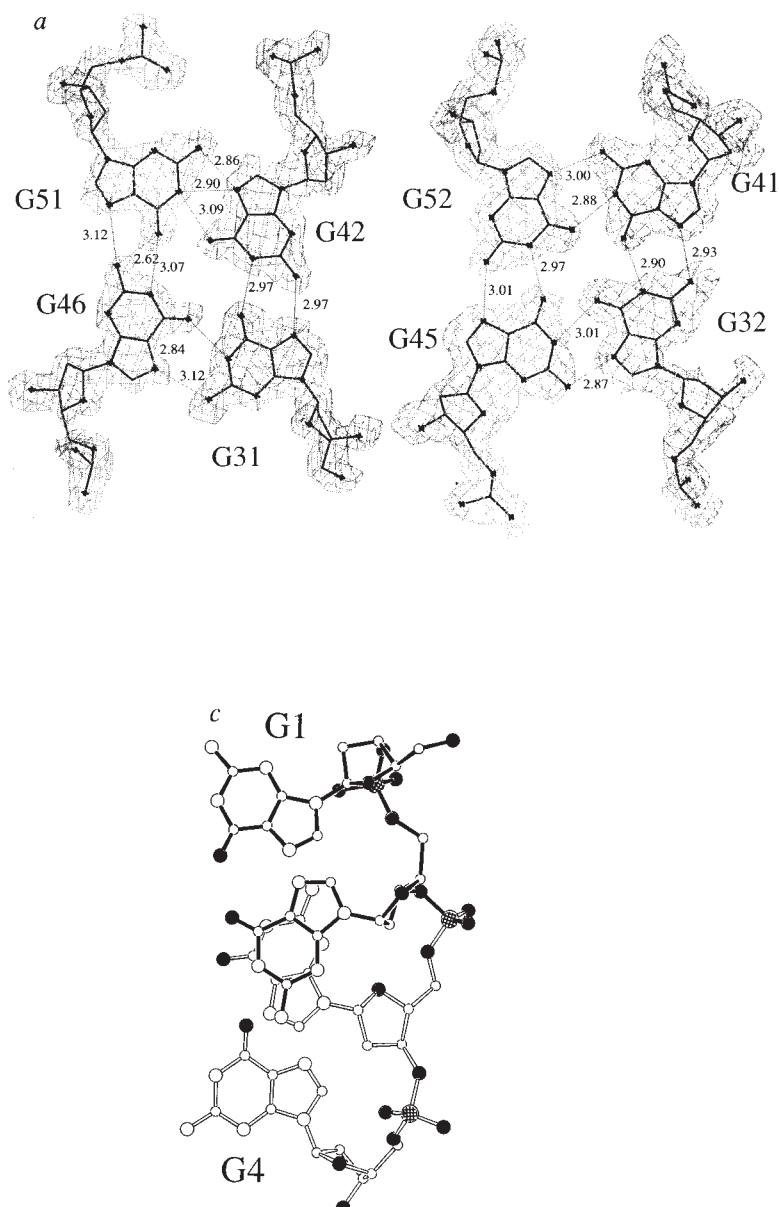
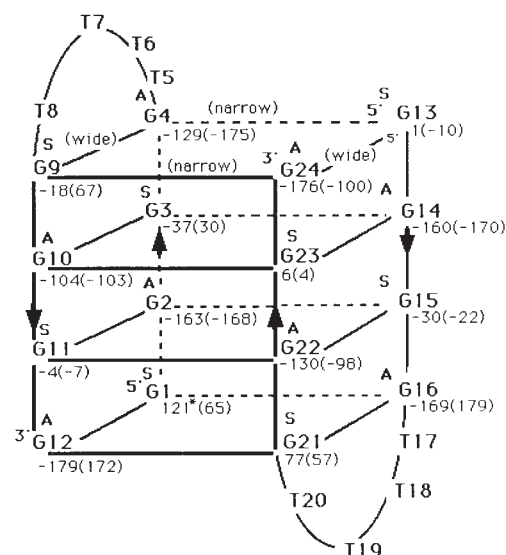


FIG. 3 The organization of guanine bases in the quadruplex. *a*, Four bases from the first two planes of the B molecule are shown in their electron density together with the system of cyclic hydrogen-bonding with bond lengths. Some hydrogen bonds are three centred as shown. The four guanines in the first plane (left) are arranged in an array that is not as square as the second plane (right). The electron density net is a $2 F_o - F_c$ map plotted at 1.0σ . *b*, A cross-section of the *Oxytricha* telomere structure of molecule A at right angles to the G quartets. The bases are shown in their electron density nets calculated at 1.0σ . Although the quartets are largely planar, there is some buckling of the bases. Stacking found in the central four G quartets is continued into some thymine residues of the loop sequence. Bases T6 and T18 are oriented parallel to the base stacking planes but are not close enough to give effective base stacking. *c*, Diagram of the four residues G1–G4 viewed perpendicular to the base planes. Guanine bases 2 and 3 are stacked on each other.

Unlike the proposed models, the guanine conformation along the chain alternate in *syn* and *anti*. Some of the explanation for this may be associated with a particularly stable interaction that is found between the bases in the two planes that have an *anti* followed by *syn* linkage. All of the four chains are similar in this regard and Fig. 3c shows a view normal to the four guanine residues in one strand. The second and third guanine residues overlap; the six-membered rings lie on top of each other and the 2-amino groups lie on top of the imidazole rings. There is considerable base stacking between planes 1 and 2 and planes 3 and 4, even though it is not as great as that found between 2 and 3. The average rotation angle between the guanine quartets is 28°.

An axial ion in the quadruplex

In the electron density map there is a peak located in the centre of the four guanines between levels 2 and 3 in both molecules. An omit map was calculated in which the central peak was not included, and the results are shown for molecules A and B in Fig. 5. The peak is slightly off-centred, somewhat closer to level 2 rather than level 3. There is a strong likelihood that this is a potassium ion in an axial position. An axial ion was initially postulated from early studies with guanylic acid gels²⁴ and polyinosinic acid²⁵. Note that the potassium ion is not well defined. When lower resolution data are included, a column of electron density is found filling the region between these two planes. Thus the potassium ion appears to be disordered in this central location.

Flexibility in the molecule

The guanine residues in the quartet planes generally fit in one plane with a small amount of buckling. But a significant exception to that is found in residue G23 as well as its pseudo symmetry-related G53, which are buckled roughly 25° from the plane of the other three guanine residues. The buckled residues are still in hydrogen-bonding distances of the other two guanines in the quartet. Two factors seem to be involved in stabilizing the buckling. The N2 group of G53 (also 23) is hydrogen-bonded to the imidazole N7 of G40 (also 10) in the normal quartet. But its N2 is also in hydrogen-bonding distance of N3 of guanine 39 (also 9) immediately above it. Thus there is a three-centred hydrogen bond from N2 of G53 (and 23) to two guanine residues in both the third and fourth quartet level. In addition, an ion or solvent molecule is coordinated to the phosphate oxygen of P53 (and 23) and P39 (also 9), both of which have rotated somewhat towards the narrow groove. That ion is also coordinated to N2 of G53 (and 23) as well as to a fourth ion or solvent molecule. The N2 amino group of G53 (and 23) is thus involved in both three-centred hydrogen bonds as well as a coordination interaction.

There are two places in which the molecules are in close van der Waals contact with other molecules in the lattice. These close contacts could be related to some of the distortion seen in the molecule and it could also be responsible for the buckling of the bases. Buckling is frequently found in double-stranded nucleic acids, and in the case of the nogalamycin-DNA complex, a G·C base pair has a 26° buckling while still hydrogen-bonded together²⁶.

Figure 5b shows the side of molecule A with the 5' end narrow groove in front. The terminal phosphates are rotated towards the narrow groove in a conformation different from that found in the other phosphate groups in the groove. Thus phosphate of G2 is hydrogen-bonded to the N2 of G15, whereas phosphates 14 and 44 are hydrogen-bonded to N2 of G3 and 33, respectively. This accounts for the marked narrowing of the groove on the 5' side of the molecule. This type of phosphate hydrogen-bonding to amino groups is also found in the structure of polyadenylic acid²⁷.

Loop conformation differences

The conformation of the four loops is best illustrated in Fig. 5, which shows two different sides of the two molecules. The loop conformations differ somewhat from each other, but there is a general organization. The first and fourth thymine bases in the loops are loosely stacked on the guanine quartets (3.7 Å), but are not in hydrogen-bonding distance. Instead, hydrogen-bonding bridging water molecules are found between the first and fourth base in three loops. The second thymine residue is aligned largely parallel and overlapping the first thymine residue, but at a spacing (4.1 Å) which is somewhat greater than found in normal stacking (Fig. 3b). The second thymine is intercalated between the third and fourth thymine and one (T6) makes a hydrogen bond with the phosphate group between the third and fourth thymine. At the apex of the loop, the third thymine residue is found in different orientations. The temperature factors (B) are significantly larger for the thymine residues than for the guanine residues, indicating significant loop mobility. Of the 16 thymine residues in the four loops, one residue, T38, appears to be in the *syn* conformation, whereas all of the remaining 15 residues are in *anti*. One ion is observed in all of the loops, positioned between the phosphates of the second and third thymine residues. These phosphates are close to each other because the chain makes a sharp turn at that point and the ion stabilizes this close interaction.

There are small but significant differences when comparing the conformation of molecule A and molecule B. The greatest difference is found near the 5' end of one strand where phosphate 3 in molecule A is rotated into the narrow groove hydrogen bonding to the 5' hydroxyl group of residue 13. But phosphate 33 in molecule B is rotated in the opposite direction into the wide groove where it hydrogen bonds to N2 of G32 in the second guanine plane. Phosphate 2 in molecule A is rotated into the narrow groove where it hydrogen bonds with N2 of G15 as mentioned above. But phosphate 32 in molecule B points out and is not involved in back hydrogen-bonding to the guanine residues. These conformational differences between molecules A and B reinforce the impression we have about the flexibility of the telomeric structure.

Discussion

This structure demonstrates that two *Oxytricha* G-rich strands can associate to form a G-quadruplex as a structural motif. This organization is generally consistent with models that have been proposed for telomere four-strand structures⁹⁻¹¹. Unlike the models, however, the sugar phosphate backbone has an alternating *syn-anti* conformation. Three guanine-containing molecules with G tracts capable of forming quartet structures, including one with the sequence studied here, have been studied by NMR¹⁷⁻¹⁹. These have all identified alternation of *anti* and *syn* conformation and our findings in this structure are consistent with those studies.

This is the second DNA structure found using alternating *anti* and *syn* conformations. In left-handed Z-DNA, the residues alternate in *anti* and *syn* conformations, with purine residues generally in *syn* conformations²⁸. In the present structure, the purine (G) residues adopt both *syn* and *anti* conformations. But the reasons for adopting alternating *syn* and *anti* conformations are not the same in the two structures. A four-stranded guanine model has been proposed in meiosis¹⁶. In that model, all four strands are parallel and the conformation of its backbone remains to be determined.

The stabilization between levels 2 and 3 in both molecules has several factors contributing to it. (1) The presence of the potassium ion between these levels; (2) the stacking overlap between guanine residues on the same chain (Fig. 3c); (3) the hydrogen-bonding between the guanine N2 groups in levels 2 and 3 (for example G3 and G15) to phosphate groups near the 5' end of the chain (P14 and P2) which are rotated into the groove to form hydrogen bonds; (4) an intricate system of

FIG. 4. Stereo view of the sugar phosphate backbone in an electron density net calculated at 0.9σ . The view shows part of the narrow groove of the A molecule with phosphates 10 (upper) and 11 on the left, phosphate 22 on the right. Rounded segments of electron density not connected to the molecule represent ions or solvent molecules.

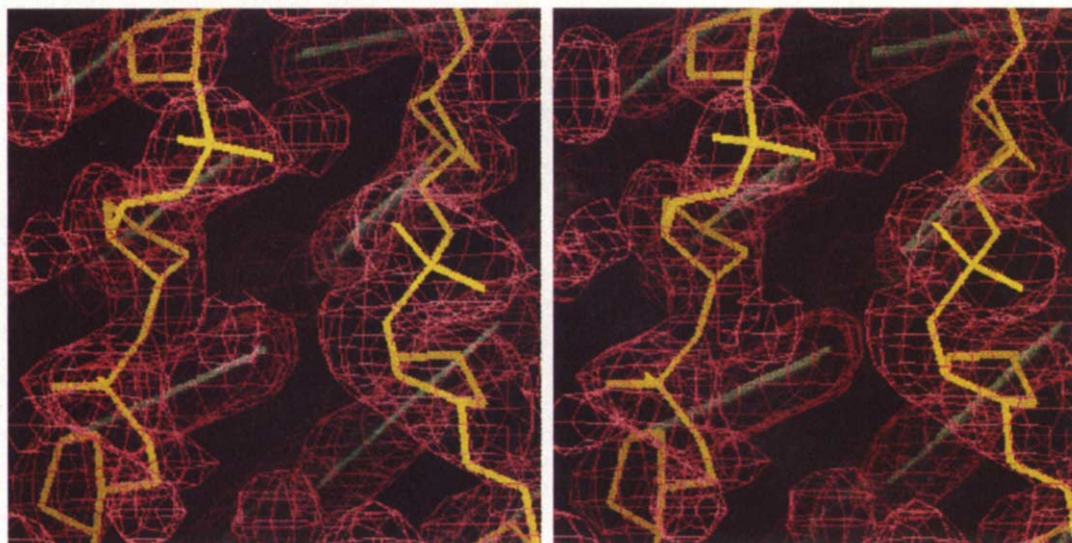
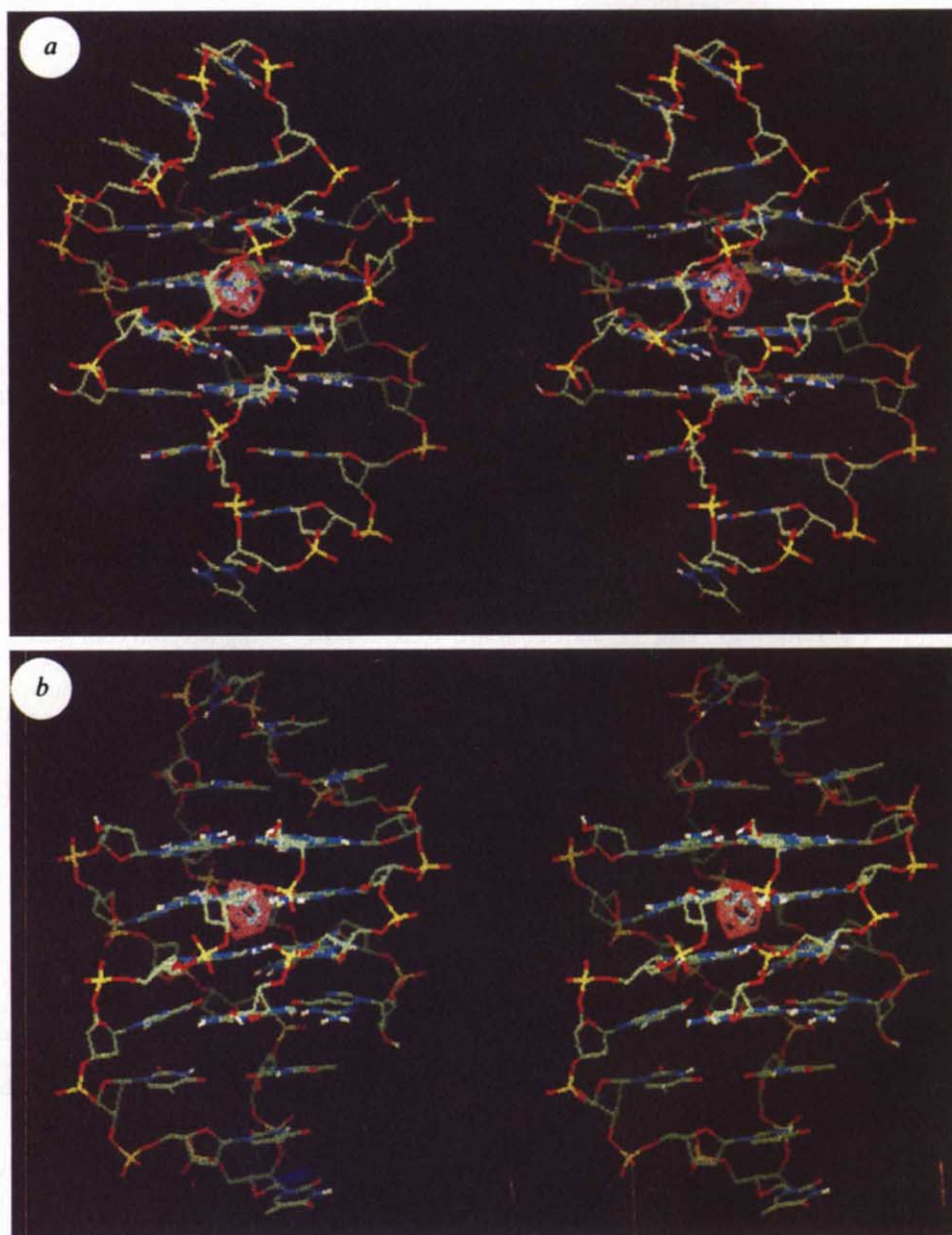


FIG. 5 The structure of the two *Oxytricha* telomere molecules in the asymmetric unit is shown together with an $(F_o - F_c)$ omit map in the central region between planes 2 and 3. The contour level of 2.5σ is red and 3.5σ is blue. *a*, Molecule A is shown with the narrow groove containing the 3' ends of the molecule at the front. *b*, Molecule B is shown with the narrow groove containing the 5' ends at the front. The phosphate at the 5' ends of both chains is missing and this narrow groove appears shorter than the groove on the other side of the molecule. In both A and B, the proximal end of the molecule closest to the pseudo 2-fold axis is at the top.



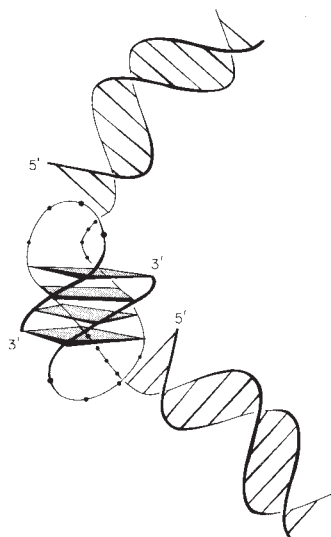


FIG. 6 A schematic diagram illustrating the manner in which the *Oxytricha* telomere structure may link together two DNA molecules or two chromosomes. The shaded squares represent the G quadruplexes and the solid dots represent the thymine residues. Small dots are also added for the four thymine residues immediately adjacent to the DNA duplex which are not found in the structure determined.

hydrogen-bonding and solvent or ion coordination. In the dimethylsulphate methylation protection experiments of Williamson *et al.*⁹, an *Oxytricha* telomere model was proposed with only two G-quartets in potassium solutions because only levels 2 and 3 gave complete methylation protection, whereas levels 1 and 4 had much less. It is probable that this result is due to the stabilization between levels 2 and 3 cited above.

The arrangement of the thymine-loop structures is generally similar to a four-base-loop structure deduced from NMR studies²⁹. The high-temperature factor and varied conformation of the thymines in the third position may be correlated with the fact that Williamson *et al.*⁹ found a photochemical crosslink between two loops in a telomeric construct in which two loops were present at the same end of the molecule. The crosslink formed between two thymine residues in the third position. The varied positions of these thymine residues might facilitate forming this photo crosslink.

Existence of four-stranded guanine complexes has not been established *in vivo*. In *Oxytricha*, the DNA fragments in the macronucleus have telomere ends with two repeats of d(T₄G₄) overhanging on the 3' end⁴. It has long been recognized that these ends form a cohering structure *in vitro*, under conditions close to physiological^{4,5,8}. This structure could be the basis for

a linear assembly of the DNA fragments in the *Oxytricha* macronucleus. Using the structure that we have found here, the organization of different DNA fragments held together by the cohering telomeres is shown diagrammatically in Fig. 6. This shows that two DNA strands come together at opposite ends of the quadruplex structure and the two telomeric repeats at the 3' end of d(T₄G₄) are folded as in the crystal. This model has been drawn to illustrate the manner in which the *Oxytricha* quartet could hold DNA molecules together; however, it could be used for holding two chromosomes together in other systems because there appears to be a common sequence organization found in all telomeres examined.

Although we have focused on telomere organizations, the structure visualized here could also have other biological roles. For example, in sister chromatid exchange, a segment of the two sister chromatid genomes containing two short G-tracts separated by other bases could form a transient four-stranded structure of this type. Its stability would compensate the fact that the normal DNA duplex must undergo strand separation for this to form. But it could be a feature of chromatid alignment. In a similar way, this structure could also be important in keeping two identical polynucleotide chains together, as for example in the two copies of the RNA genome present in all retroviruses, including human immunodeficiency virus³⁰. □

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- Blackburn, E. *Nature* **350**, 569-572 (1991).
- Klobutcher, L. A., Swanton, M. T., Donini, P. & Prescott, D. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3015-3019 (1981).
- Henderson, E. R. & Blackburn, E. H. *Molec. cell. Biol.* **9**, 345-348 (1989).
- Lipps, H. J., Grisssem, W. & Prescott, D. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2495-2499 (1982).
- Oka, Y. & Thomas, C. A. *Nucleic Acids Res.* **15**, 8877-8898 (1987).
- Henderson, E., Hardin, C. C., Walk, S. K., Tinoco, I. & Blackburn, E. H. *Cell* **51**, 899-908 (1987).
- Cech, T. R. *Nature* **332**, 777-778 (1988).
- Lipps, H. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4104-4107 (1980).
- Williamson, J. R., Raghuraman, M. K. & Cech, T. R. *Cell* **59**, 871-880 (1990).
- Sundquist, W. I. & Klug, A. *Nature* **342**, 825-829 (1990).
- Panyutin, I. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 867-870 (1990).
- Rich, A. *Biochim. biophys. Acta* **29**, 502-509 (1958).
- Arnott, S., Chandrasekaran, R. & Marttila, C. M. *Biochem. J.* **141**, 537-543 (1974).
- Zimmerman, S. B., Cohen, G. H. & Davies, D. R. *J. molec. Biol.* **92**, 181-192 (1975).
- Gellert, M., Lipsett, M. N. & Davies, D. R. *Proc. natn. Acad. Sci. U.S.A.* **48**, 2013-2018 (1962).
- Sen, D. & Gilbert, W. *Nature* **334**, 364-366 (1988).
- Wang, Y., Jin, R., Gaffney, B., Jones, R. A. & Breslauer, K. J. *Nucleic Acids Res.* **19**, 4619-4622 (1991).
- Wang, Y. *et al. J. molec. Biol.* (in the press).
- Smith, F. W. & Feigon, J. *J. biomolec. Struct. Dynamics* **8**, 201 (1991).

- Brunger, A. T. *Acta crystallogr. A* **46**, 46-57 (1990).
- Zimmerman, S. B. *J. molec. Biol.* **106**, 663-672 (1976).
- Brunger, A. T. *X-PLOR Manual, Version 2.1* Yale University (1990).
- Jones, T. A. *J. appl. Crystallogr.* **11**, 268-276 (1978).
- Pinnaraia, T. J. *et al. J. Am. chem. Soc.* **100**, 3625-3627 (1978).
- Howard, F. B. & Miles, H. T. *Biochemistry* **21**, 6736-6745 (1982).
- Williams, L. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 2225-2229 (1990).
- Rich, A., Davies, D. R., Crick, F. H. C. & Watson, J. D. *J. molec. Biol.* **3**, 71-86 (1961).
- Rich, A., Nordheim, A. & Wang, A. H.-J. *A. Rev. Biochem.* **53**, 791-846 (1984).
- Hare, D. R. & Reid, B. R. *Biochemistry* **25**, 5341-5350 (1986).
- Kung, H. J. *et al. Cell* **1**, 609-620 (1976).

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Two-population model for the sources of γ -ray bursts

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RECENT measurements by the Burst and Transient Source Experiment (BATSE) on the Compton Gamma Ray Observatory Satellite suggest that the distribution of γ -ray burst sources is isotropic about the Earth's position but also radially non-uniform¹. Studies of their spectral and temporal properties², however, suggest that many bursts originate on or near neutron stars, presumed to be part of the local galactic population. We show here that no single class of bursts, either galactic or cosmological in origin, can explain all of these observations, but argue instead that the constraints can be reconciled if there are at least two distinct classes of bursts. As a specific example we consider two kinds of burst which both originate on galactic neutron stars, but which differ in intrinsic luminosity by a factor of 10^5 .

A neutron-star origin of the bursts is strongly suggested² by observations of absorption lines at 20–50 keV, attributed to cyclotron scattering and absorption in 10^{12} -G magnetic fields; by emission lines seen at 350–500 keV, attributed to gravitationally redshifted electron–positron annihilation radiation with neutron-star redshifts of 0.2 or more; by the very small source sizes (<60 km) implied by burst rise times as short as 0.2 ms; by the very high mean density of $>2 \times 10^6$ g cm⁻³ implied for solid-body rotation of the source, if the 8-s period in the late-time emission of the burst GB790305b is rotational; and by the location of the latter source position within the supernova remnant N49 in the Large Magellanic Cloud. Local galactic distances (<0.6 kpc) are also suggested by the black-body limits on the recently detected³ soft X-ray emission from bursts and by the limits set by features of cyclotron line absorption⁴. These spectral and temporal constraints cannot be neglected in the interpretation of the spatial distribution.

The BATSE measurements¹ of the distribution of the observed burst intensities and their positions on the sky seem to be inconsistent with a local galactic neutron-star origin. Various size–frequency distributions have been used in the past to look for non-uniformity in the spatial distribution of γ -ray bursts, but these can suffer from serious selection biases⁵. The least biased and most sensitive measure is $V/V_{\max}$, which determines⁶ for each burst the ratio of the volume defined by the distance of the burst and the maximum volume from which the burst could have been detected by the instrument at that time. If the source population is spatially uniform, then in a euclidean geometry, the values of $V/V_{\max}$ will be uniformly distributed between 0 and 1, and the mean value of $V/V_{\max}$ is 0.5, with a minimum root-mean-square error of $(12N)^{-1/2}$, where N is the number of bursts in the sample. If the sources are galactic and their bursts could be detected at distances greater than their scale size, then $\langle V/V_{\max} \rangle$ would be <0.5.

Constraints^{7,8} on the source distribution can also be obtained from measurements of their distribution of the sky, particularly the dipole and quadrupole moments of the source directions for a galactic distribution. The dipole moment is proportional to $\langle \cos \theta \rangle$, where θ is the angle of the source with respect to the Galactic Centre, and the quadrupole moment is essentially proportional to $\langle \sin^2 b \rangle - 1/3$, where b is the galactic latitude. If the distribution of detectable sources is uniform, the mean $\langle \cos \theta \rangle$ is 0, and $\langle \sin^2 b \rangle$ is 1/3.

An analysis of the bursts detected by BATSE now shows¹ $\langle V/V_{\max} \rangle = 0.348 \pm 0.024$, which is significantly less than 1/2. But the observed distribution of these bursts on the sky is

apparently isotropic, with $\langle \cos \theta \rangle = 0.01 \pm 0.05$ quite consistent with 0 and $\langle \sin^2 b \rangle = 0.31 \pm 0.024$ consistent with 1/3. These measurements seem to contradict a local galactic neutron-star origin of the bursts, because calculations⁸ of both galactic disk and halo populations show that for $\langle V/V_{\max} \rangle = 0.348 \pm 0.024$, as observed by BATSE, the expected anisotropy should be much greater than measured limits. For a simple disk population, $\langle V/V_{\max} \rangle$ asymptotically approaches 0.40 as $\langle \sin^2 b \rangle$ goes to 0, so that the BATSE 2σ lower limit on $\langle \sin^2 b \rangle$ of 0.26 would not be consistent with $\langle V/V_{\max} \rangle$ less than ~ 0.46 . Similarly for a halo population of high-velocity neutron stars, a $\langle V/V_{\max} \rangle$ of 0.348 ± 0.024 would require a $\langle \cos \theta \rangle$ of ~ 0.46 , far above the BATSE 2σ upper limit of 0.11. Generic halo populations have also been studied⁹ with neutron star densities proportional to $[1 + (R/R_c)^\alpha]^{-1}$ where R is the galactic radial distance, R_c is the core radius, and α is equal to 3 if the distribution is similar to that for luminous matter, and 2 for dark matter. Such a population would be consistent with both the observed $\langle V/V_{\max} \rangle$ and $\langle \cos \theta \rangle$ if R_c was >20 kpc and the maximum distance at which a burst can be detected $d_{\max}$ was a few times larger.

But in such a distant population alone the probability of observing a source closer than a distance $d < R_c$ is of the order of $(d/R_c)^3$, independent of α , or $<10^{-4.5}$ for $d < 0.6$ kpc and $R_c > 20$ kpc, which is not consistent with the observation of soft X-ray emission³ and cyclotron absorption features⁴ in the spectra of several bursts in a sample of less than 100. This inconsistency is magnified by many orders of magnitude for any much more distant cosmological source. Moreover, cosmologically distant source populations alone are inconsistent with the observed spectral and temporal properties of γ -ray bursts, which strongly suggest^{2–4} a neutron-star origin for many of the bursts, and models in which energy is derived from mergers of a neutron star with a black hole¹⁰ obviously cannot account for the repeating bursts which make up at least 130 of the ~ 650 observed bursts². No single class of burst sources suggested so far, whether galactic or cosmological, can account for all of the observed properties of γ -ray bursts. Considering the enormous diversity of observed burst spectra, durations and time variations, however, there is no reason to expect that all of the bursts arise from a single class of sources.

We suggest, therefore, that these seemingly conflicting constraints can be reconciled if the observed γ -ray bursts come from two or more distinct source populations. Such a solution was also suggested¹¹ for the excess detected by the Solar Maximum Mission at low $V/V_{\max}$. Two galactic populations can provide a consistent solution where one will not, because, as mentioned above, theoretical calculations^{7–9} have shown that only galactic populations observed at $\langle V/V_{\max} \rangle$ close to either 0.5 or 0 can have values of $\langle \cos \theta \rangle$ and $\langle \sin^2 b \rangle$ within current observational¹ limits, whereas those observed at intermediate values of $\langle V/V_{\max} \rangle$ are anisotropic, greatly exceeding current limits. Two populations, one observed at $\langle V/V_{\max} \rangle \approx 0$ and the other at $\langle V/V_{\max} \rangle \approx 0.5$, can be combined to give an intermediate value of $\langle V/V_{\max} \rangle$ and still be within current limits on isotropy, as the mean values of $\langle V/V_{\max} \rangle$, $\langle \cos \theta \rangle$ and $\langle \sin^2 b \rangle$ are all additive.

In particular, an extended population of highly luminous bursts, observed to distances well beyond their effective scale size, could provide the very low $\langle V/V_{\max} \rangle$ component, and a more local population of much less luminous bursts, observed only to distances much less than their effective scale size, could provide the nearly uniform $\langle V/V_{\max} \rangle$ component that is also essentially isotropic within current limits. Alternatively, a very local population, lying well within the detection limit, concentrated for example in the Gould Belt (J.C.H., manuscript in preparation), could provide the very low $\langle V/V_{\max} \rangle$ component and possibly be within the anisotropy limits, and a more distant galactic population with a scale size beyond the detection limit could provide the nearly uniform $\langle V/V_{\max} \rangle$ component.

Thus two populations offer a generic solution to the apparent

contradiction between the observations and galactic neutron-star populations. Such a model can be consistent not only with the spatial distribution constraints, but also with all of the spectral and temporal constraints, which no single population can accommodate.

Here we will consider two distinct luminosity classes, and leave further discussion of a possible local concentration to a future paper (J.C.H., manuscript in preparation). As a simple example, we assume that both populations arise on a single spatial distribution of neutron stars with some characteristic scale size d_{ns} , and that two different mechanisms produced bursts with very different mean luminosities, $\langle L \rangle_{\text{low}} \ll \langle L \rangle_{\text{high}}$. Because $d_{\text{max}} \propto L^{-1/2}$, the relative distances are $\langle d_{\text{max}} \rangle_{\text{low}} \ll d_{\text{ns}} \ll \langle d_{\text{max}} \rangle_{\text{high}}$ and the corresponding relative volumes are $\langle V/V_{\text{max}} \rangle_{\text{high}} \ll \langle V/V_{\text{max}} \rangle_{\text{low}}$. For illustration, we consider two possible burst mechanisms: thermonuclear runaway of accreted matter on the polar caps of neutron stars which might release energies of $\sim 10^{39}$ erg and make up the low-luminosity class, and neutron star quakes which might release energies of $\sim 10^{44}$ erg and make up the high-luminosity class. With a luminosity ratio of $\sim 10^5$ between the high- and low-luminosity classes, their limiting detection distances differ by $10^{2.5}$. For typical bursts, these luminosities would correspond to a nominal d_{max} of 0.3 kpc for the low-luminosity bursts and 100 kpc for the high-luminosity bursts. Then if we consider as sources the galactic high-velocity neutron-star population for which there are published⁸ calculations of the $\langle V/V_{\text{max}} \rangle$, $\langle \cos \theta \rangle$ and $\langle \sin^2 b \rangle$ as a function of d_{max} , we find that the high-luminosity bursts would have $\langle V/V_{\text{max}} \rangle$ of 0.20, $\langle \cos \theta \rangle$ of 0.19 and $\langle \sin^2 b \rangle$ of 0.18, and the low-luminosity bursts would have corresponding values of 0.49, 0.02 and 0.31, respectively. If the high-luminosity class makes up 1/3 of the observed bursts, then the combination of the two classes would give an expected values of $\langle V/V_{\text{max}} \rangle$ of 0.39, $\langle \cos \theta \rangle$ of 0.08 and $\langle \sin^2 b \rangle$ of 0.27, which are all consistent within 1.7, 1.4 and 1.7σ of the values of 0.348 ± 0.024 , 0.01 ± 0.05 and 0.31 ± 0.024 observed by BATSE. Such anisotropies should be measurable by BATSE within a year or two.

These measurements can then be compared with much more realistic calculations of the $\langle V/V_{\text{max}} \rangle$ and anisotropies expected from two-population models in which the distribution of burst sources is not simply the spatial distribution of the neutron stars. For bursts from the thermonuclear runaway of accreted interstellar matter, the distributions of interstellar gas, density and the neutron-star velocities and magnetic field strengths, which determine the accretion rate, will also affect both the burst rate and the resulting luminosity distribution¹², as a function of distance above the galactic plane. Similarly for bursts from neutron-star quakes driven by spin-down, the distribution of neutron-star ages will affect both the burst rate and the resulting luminosity distribution as a function of galactic distance. And this, of course, is just one possible combinations of different populations.

It is tempting to try to test¹³ two-population and other models by comparing the burst sample seen by BATSE with those found by other burst experiments. A range of values of $\langle V/V_{\text{max}} \rangle$ have been reported: 0.40 ± 0.03 from the Solar Maximum Mission¹¹, 0.43 ± 0.024 from Konus (including the <1 -s bursts¹⁴, excluded by Higdon and Schmidt¹⁵), 0.46 ± 0.024 from Pioneer Venus Orbiter (PVO)¹⁶, 0.43 ± 0.024 from Signe, Apex and Lilas¹⁷, and 0.35 ± 0.035 from Ginga¹⁸; nearly all of these values are significantly less than 0.5. But even the difference between the extreme values, 0.348 ± 0.024 from BATSE¹ and 0.46 ± 0.024 from PVO¹⁶, is only significant at the 3σ level in the statistical errors alone, and consideration of systematic uncertainties will make that less.

Even if significant differences can be established, comparisons between experiments, which have different energy- and time-dependent detection thresholds, are not simple. For example, the relative number of bursts observed by a particular detector

from two different populations will depend not only on the assumed mean ratio of intrinsic luminosities, discussed above, but also on differences in the mean spectra, durations and time profiles, which all determine whether a given burst will be detected by different detectors sampling on different energy bands, timescales and trigger criteria. Such selection effects are important even for the brightest bursts, as can be seen by the fact that the PVO experiment, triggering on the flux in the 100-keV to 2-MeV band on a 0.25-s integration (K. W. Chuang, personal communication), saw only about half (11 out of 19) of the brightest ($V/V_{\text{max}} < 0.1$) bursts detected by Konus¹⁴ in the 50–150 keV band on the same integration time. We cannot compare burst samples from different detectors with any confidence until these different competing effects are understood and taken into account. $\square$

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1. Meegan, C. et al. *Nature* **355**, 143–145 (1991).
2. Higdon, J. C. & Lingelfelter, R. E. *Astr. Astrophys.* **28**, 401–436 (1990).
3. Murakami, T. et al. *Nature* **350**, 592–594 (1991).
4. Lamb, D., Wang, J. C. L. & Wasserman, I. M. *Astrophys. J.* **363**, 670–693 (1990).
5. Higdon, J. C. & Lingelfelter, R. E. *Astrophys. J.* **307**, 197–204 (1986).
6. Schmidt, M., Higdon, J. C. & Hueter, G. *Astrophys. J.* **329**, L85–87 (1988).
7. Hartmann, D., Epstein, R. I. & Woosley, S. E. *Astrophys. J.* **348**, 625–633 (1990).
8. Paczynski, B. *Astrophys. J.* **348**, 485–494 (1990).
9. Paczynski, B. *Acta astr.* **41**, 157–166 (1991).
10. Paczynski, B. in *Huntsville Workshop on Gamma Ray Bursts* (eds Paciesas, W. & Fishman, G.) (American Institute of Physics, New York, in the press).
11. Matz, S. et al. in *Gamma Ray Bursts* (eds Ho, C., Fenimore, E. E. & Epstein, R. I.) (Cambridge University Press, in the press).
12. Higdon, J. C. & Lingelfelter, R. E. in *High Energy Transients in Astrophysics* (ed. Woosley, S. E.) 568–577 (American Institute of Physics, New York, 1984).
13. Mao, S. & Paczynski, B. *Astrophys. J.* (submitted).
14. Mazets, E. et al. *Astrophys. Space Sci.* **80**, 1–143 (1982).
15. Higdon, J. C. & Schmidt, M. *Astrophys. J.* **355**, 13–17 (1990).
16. Hartmann, D. et al. in *Gamma Ray Bursts* (eds Ho, C., Fenimore, E. E. & Epstein, R. I.) (Cambridge University Press, in the press).
17. Atteia, J. L. et al. *Nature* **351**, 296–298 (1991).
18. Ogasaka, Y., Murakami, T., Nishimura, J., Yoshida, A. & Fenimore, E. E. *Astrophys. J.* (submitted).

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Defect-induced melting and solid-state amorphization

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DESPITE the strong interest in melting over the past 100 years, a general theory for the crystal–liquid transition has not been established¹. Lattice-instability models, which are either vibrational², elastic³, isochoric⁴, defective⁵ or entropic⁶ in nature, all predict a melting point somewhat above the experimentally observed thermodynamic melting temperature, with the ultimate stability limit of a superheated crystal being determined by the equality of crystal and liquid entropies^{4,6}; this forces regular melting to be a first-order transition. Here I present a model of melting that is driven by the incorporation into the lattice of randomly frozen-in defects. An isentropic condition limits the stability of the crystal as a function of defect concentration; above the glass transition temperature the crystal melts to a liquid, whereas below it ‘melting’ produces an amorphous solid. This model yields a generic melting diagram with a tunable parameter (defect concentration) that can characterize the static disorder present in solid-state amorphization^{7–9}, the thermodynamic stability of small clusters¹⁰ and nanocrystalline materials¹¹, and the frustration present in spin glasses¹². The model is also relevant to glacial¹³, geological¹⁴ and stellar-atmospheric¹⁵ melting processes.

A crystal can be ‘melted’ to an amorphous solid when a thermodynamic driving force for the transition develops and kinetic constraints are maintained (mainly because of the low experimental temperatures) that prevent the establishment of

full equilibrium^{7,8}. Such a transition can be described by standard thermodynamic methods if the relevant kinetic constraints are defined appropriately. In particular, we want to consider the destabilization of the crystalline lattice by the incorporation of nonequilibrium point defects, such as vacancies, anti-site defects and interstitials, typical for externally driven amorphization reactions, such as irradiation and mechanical deformation. By comparing the Gibbs free energy of the undercooled liquid with that of the crystal containing point defects (nonequilibrium melting), the relative stability of such nonequilibrium structures can be evaluated by the following universal relationships. The free energy difference $\Delta G = \Delta H - T\Delta S$ (ΔH and ΔS denote the enthalpy and entropy differences, respectively) between a liquid below its equilibrium melting point and the corresponding single crystal can be realistically approximated for pure metals^{16,17} by $\Delta G = 7\Delta S_f \Delta T \cdot T / (T_m + 6T)$ and for glass-forming metallic alloys^{18,19} by $\Delta G = 2\Delta S_f \Delta T \cdot T / (T_m + T)$, where ΔT is the undercooling below the melting point T_m and ΔS_f the entropy of fusion (typically $1.1k_B$ for pure metals and $1.5k_B$ for binary intermetallic compounds²⁰). The free energy increase of the crystalline phase is obtained as a function of point defect concentration c^v as $\Delta G^v = c^v(\Delta H^v - T\Delta S^v) + k_B T(c^v \ln c^v + (1 - c^v) \ln(1 - c^v))$ ²¹, with the last term corresponding to the ideal configurational entropy.

As a typical example, we consider vacancies, with the heat of formation of a vacancy, ΔH^v , being related to the equilibrium melting point T_m by a universal relationship established for a wide range of metals, where $\Delta H^v = 8 \times 10^{-4} T_m$ eV per Kelvin per atom (or $9.28k_B T_m$)²² and the entropy of formation of a vacancy²¹, ΔS^v , is about $2k_B$. This approach works equally well if we consider other point defects such as anti-site defects and interstitials.

For a glass-forming alloy, the Gibbs free energy functions of the undercooled liquid and the nonequilibrium crystal are shown in Fig. 1 as a function of temperature and vacancy concentration with the ideal single crystal as reference ($G=0$). The virtual melting point of the defective crystal can be determined as a function of vacancy concentration by the equality of the crystal and liquid Gibbs free energies ($\Delta G^* = \Delta G - \Delta G^v = 0$) under the assumption that the defects remain frozen in the lattice. Such an assumption is fully justified, especially at low temperatures; it is well known that γ -irradiation lowers the melting points of pure metals by an amount that is proportional to the dose, and thus to the number of point defects generated^{1,5}.

In addition, the entropy and enthalpy differences ΔS^* and ΔH^* between undercooled liquid and crystal containing defects can be obtained from simple thermodynamic relationships as

$\Delta S^* = -\partial \Delta G^* / \partial T$ and $\Delta H^* = \Delta G^* + T\Delta S^*$. With increasing undercooling, ΔS^* generally decreases faster than ΔH^* . In Fig. 1 the isentropic temperature, T_{g0} , is indicated where the slope of the liquid free energy equals that of the ideal crystal. Below this temperature the liquid state would have a smaller entropy than the crystalline state. According to Kauzmann²³, such a catastrophe is avoided through massive freezing of the liquid to a glass at or above T_{g0} , and thus the isentropic temperature is usually not reached. On the other hand, an 'inverse' Kauzmann paradox can be applied to a crystal superheated by the incorporation of nonequilibrium point defects. Accordingly, the superheated crystal becomes unstable and melts when the free energy and entropy both become larger than that of the liquid phase⁶. As indicated in Fig. 1, this condition is met at a critical vacancy concentration c_v^* and temperature T^* . As such, a crystal with vacancy concentration larger than c_v^* is strictly unstable.

On the basis of the universality of the free energy relationships, a generic nonequilibrium melting diagram can be developed as shown in Fig. 2, indicating a decrease of the virtual melting point as a function of vacancy concentration. This melting curve, with slope $dT/dc_v = -(\partial \Delta G^* / \partial c_v) / \Delta S_f$, is related to the T_0 lines in binary phase diagrams^{6,24}. Also included are the isentropic and isenthalpic temperatures as a function of vacancy concentration. It can be seen that the melting ($\Delta G^* = 0$), isentropic ($\Delta S^* = 0$) and isenthalpic lines ($\Delta H^* = 0$) must intersect at a temperature T^* and vacancy concentration c_v^* . At this condition, melting is reduced to an isentropic transition with $\Delta G^* = \Delta S^* = \Delta H^* = 0$ (no latent heat) because the configurational entropy of crystal plus defects equals the liquid entropy. Such a point is not unusual in phase diagrams and has been predicted by applying scaling and renormalization group theories²⁵ to experimental results for the amorphization of crystalline metal hydrides²⁶, as a thermodynamic critical fixed point underlying the glass transition.

The slope of the melting curve increases with c_v and reaches infinity at the melting instability ($\Delta S_f = \Delta S^* = 0$). Within our simple model, this melting instability is predicted at $c_v^* = 0.077$ and $T^* = 0.52 T_m$ for intermetallic compounds exhibiting solid-state amorphization, and at $c_v^* = 0.077$ and $T = 0.35 T_m$ for pure metals, respectively. In general, such values of c_v for metals and most intermetallic compounds are far beyond equilibrium values (typically 10^{-3} close to T_m) and are a measure for the deviation from internal equilibrium. Below T^* , the melting curve bends back, and the entropy of fusion would become negative and, thus, melting exothermic.

Even though glass formation is a dynamic process, with the actual glass transition temperature being characterized by loss

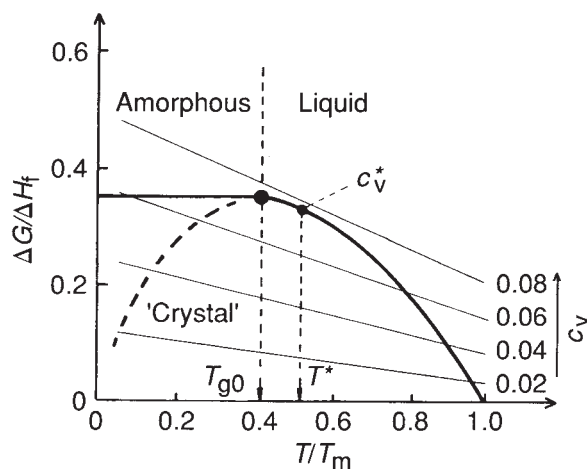


FIG. 1 Gibbs free energy difference ΔG (thick line) between the undercooled liquid and an ideal single crystalline intermetallic compound ($G=0$), together with the Gibbs free energy increase of the crystalline state ΔG^v (thin line) with a lattice vacancy content c_v . The undercooled liquid is distinguished from the amorphous solid by the ideal glass-transition temperature T_{g0} .

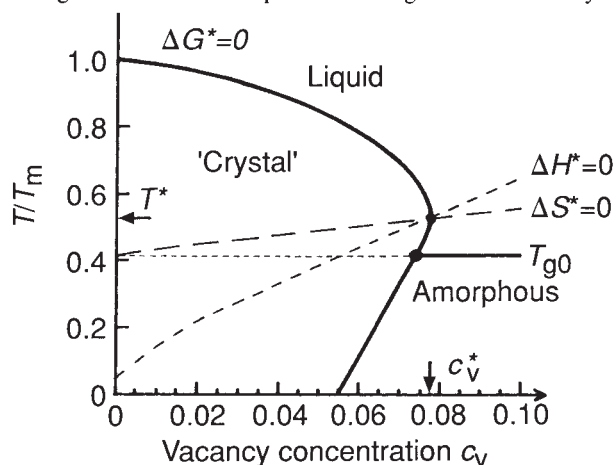


FIG. 2 Universal melting diagram representing three nonequilibrium states: the crystal supersaturated with vacancies, the undercooled liquid and the amorphous solid. Additionally included as a function of the respective crystal defect concentrations, c_v , are the isentropic ($\Delta S^* = 0$) and isenthalpic temperatures ($\Delta H^* = 0$) which cross the melting line ($\Delta G^* = 0$) at the melting instability (T^* , c_v^*).

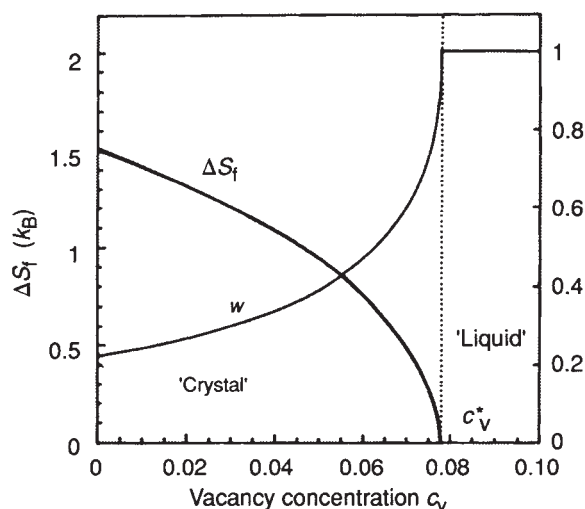


FIG. 3 *a*, Entropy difference ΔS_f along the melting line above T^* . *b*, Probability $w = \exp(-\Delta S_f/k_B)$ for heterophase fluctuations of liquid-like clusters in the defective crystal close to the virtual melting point.

of ergodicity and the internal relaxation times (viscosity) strongly depending on the cooling rate, the isentropic condition, where the entropies of liquid and ideal crystal are equal, agrees reasonably well with measured glass transition temperatures¹⁹. Below T_{g0} the liquid is replaced by an amorphous solid which in the configurationally frozen state preserves the enthalpy and entropy values of the liquid at T_{g0} . This behaviour below T_{g0} is reflected by the constant free energy level indicated in Fig. 1 and by the corresponding temperature dependence of the extended 'melting' curve in Fig. 2, implying an exothermic reaction during solid-state amorphization (re-entrant melting²⁷).

In this model, a crystal can be melted to a liquid by an increase of temperature or to an amorphous solid by the incorporation of nonequilibrium point defects. But by the time that the vacancy (or other defect) concentration is so high that the nose of the $\Delta G^* = 0$ curve has been reached, the entropy of the defective crystal will be well above that of the ideal crystal. As such, the isentropic line of Fig. 2 lies significantly above the ideal glass transition line according to Kauzmann. This situation is different from binary solid solutions where the Kauzmann condition coincides with the melting instability⁶.

To analyse the melting transition further, the entropy of fusion along the upper part of the melting curve is represented in Fig. 3 by curve *a*, ranging from $\Delta S_f = 1.5k_B$ at T_m to $0k_B$ at the melting instability (c_v^* , T^*). The kinetics of melting depend strongly on the development of heterophase fluctuations, represented by small liquid-like clusters evolving in the crystalline phase and breaking the symmetry of the crystalline lattice²⁸. For a closed system, such fluctuations, *w*, occur near the melting point with probability $\exp(-\Delta S_f/k_B)$ ²⁹. As such, they are greatly enhanced with increasing vacancy concentration, as shown by curve *b* in Fig. 3. Because the entropy of fusion ΔS_f vanishes for the supersaturated crystal at c_v^* , the probability for liquid-like heterophase fluctuations diverges and reaches one at c_v^* . Under this condition, the crystal melts by an intrinsic instability (entropy catastrophe) which has been predicted as the ultimate stability limit of a superheated crystal⁶.

This approach is a general one and applies to many nonequilibrium structures. For example, the number of vacancies basically corresponds to the number of broken bonds in a lattice. As such, the vacancies could condense to free surfaces, forming small metal clusters¹⁰ with the surface energy γ being proportional to the heat of formation of a vacancy³⁰, or to high-angle grain boundaries, forming nanocrystalline material¹¹. This is consistent with experimental observations on melting of small metal clusters of, for example, gold, indicating a marked depression of melting point with smaller cluster size¹⁰. This reduces the melting point to $\sim 1/3$ of its bulk value at a critical diameter of 25 Å for gold clusters. At this condition ($T^* = 0.33 T_m$), the slope of the melting line approaches infinity, in agreement with the

predicted diagram in Fig. 2. For particle sizes smaller than 25 Å, the system approaches a quasi-liquid state fluctuating between the crystalline and fluid structure¹⁰.

Consequently, the first-order melting transition characterized by a discontinuous increase of vacancy concentration c_v from typically 10^{-3} (crystal at T_m) to 10^{-1} (liquid) in a vacancy model⁵ can be reduced to continuous-like behaviour by the incorporation of randomly frozen-in lattice vacancies at $c_v^* = 0.077$. Furthermore, the vacancy concentration described here represents a general, tunable parameter for the static disorder or degree of frustration of the system, resulting in a universal nonequilibrium melting diagram with strong similarities to the magnetic spin-glass transition¹². The level of disorder could correspond to the concentration of point defects (vacancies, interstitials and/or anti-site defects), alloy composition, pressure, excess free volume, internal stress, reciprocal particle size in the case of small clusters and/or reciprocal grain size in case of nanocrystalline materials. Above a certain degree of frustration (c_v^*), the crystalline state becomes intrinsically unstable against liquid-like heterophase fluctuations and the system collapses to a disordered state, as indicated by the intrinsic melting instability with intersecting isentropic, isenthalpic and isenergetic lines. □

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1. Cahn, R. W. *Nature* **323**, 668–669 (1986).
2. Lindemann, F. A. *Z. Phys.* **11**, 609–615 (1910).
3. Born, M. *J. chem. Phys.* **7**, 591–599 (1939).
4. Tallon, J. L. *Nature* **342**, 658–660 (1989).
5. Gorecki, T. *Scripta metall.* **11**, 1051–1057 (1977).
6. Fecht, H. J. & Johnson, W. L. *Nature* **334**, 50–51 (1988).
7. Johnson, W. L. *Progr. Mat. Sci.* **30**, 81–134 (1986).
8. Samwer, K. *Phys. Rep.* **161**, 1–41 (1988).
9. Wolf, D., Okamoto, P. R., Yip, S., Lutsko, J. F. & Kluge, M. *J. Mat. Res.* **5**, 286–301 (1990).
10. Ajavan, P. M. & Marks, L. D. *Phys. Rev. Lett.* **60**, 585–587 (1988).
11. Gleiter, H. *Prog. Mat. Sci.* **33**, 223–315 (1989).
12. LeDoussal, P. & Harris, A. B. *Phys. Rev. Lett.* **61**, 625–629 (1988).
13. Mishima, O., Calvert, L. D. & Whalley, E. *Nature* **310**, 393–395 (1984).
14. Richet, P. *Nature* **331**, 56–57 (1988).
15. Kouchi, A. & Kuroda, T. *Nature* **344**, 134–136 (1990).
16. Singh, H. B. & Holz, A. *Solid State Comm.* **45**, 985–987 (1983).
17. Perepezko, J. H. & Paik, J. S. *J. non-cryst. Sol.* **61** & **62**, 113–116 (1984).
18. Thompson, C. V. & Spaepen, F. *Acta metall.* **27**, 1855–1859 (1979).
19. Fecht, H. J., Perepezko, J. H., Lee, M. C. & Johnson, W. L. *J. appl. Phys.* **68**, 4494–4502 (1990).
20. Goodman, D., Cahn, J. W. & Bennett, L. H. *Bull. Alloy Phase Diag.* **2**, 29–35 (1981).
21. Wollenberger, H. J. in *Physical Metallurgy* (eds Cahn, R. W. & Haasen, P.) 1139 (Elsevier, Amsterdam, 1983).
22. Doyama, M. & Koehler, J. S. *Acta Metall.* **24**, 871–879 (1976).
23. Kauzmann, W. *Chem. Rev.* **43**, 219–256 (1948).
24. Fecht, H. J., Fu, Z. & Johnson, W. L. *Phys. Rev. Lett.* **64**, 1753–1756 (1990).
25. Sethna, J. P., Shore, J. D. & Huang, M. *Phys. Rev. B* **44**, 4943–4959 (1991).
26. Fecht, H. J., Desr  , P. & Johnson, W. L. *Phil. Mag.* **B59**, 577–585 (1989).
27. Highmore, R. J. & Greer, A. L. *Nature* **339**, 363–365 (1989).
28. Yukalov, V. I. *Phys. Rev. B* **32**, 436–446 (1985).
29. Lifshitz, E. M. & Pitaevskii, L. P. *Statistical Physics*, 3rd edn (Pergamon, Oxford, 1980).
30. Gorecki, T. *Z. Metallkunde* **70**, 121–126 (1979).

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A modified cyclodextrin as a guest responsive colour-change indicator

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CHEMICAL indicators that change colour in response to the presence of neutral organic molecules are valuable for qualitative chemical analysis. Although some ionophores are known to exhibit colour changes on binding metal or ammonium cations^{1–3}, however, the detection of neutral organic species in solution by this means remains problematic. We have shown recently^{4–7} that some fluorophore-modified cyclodextrins exhibit variations in fluorescence intensity on binding organic guests. Here we report guest-selective binding to a cyclodextrin that has been modified in such a way as to undergo a colour change on host–guest complexation. We attach the pH indicator methyl red to the wall of a β -cyclodextrin: in acidic solution, the modified cyclodextrin remains yellow owing to binding of the methyl red group inside the cavity, protecting it from protonation. When an organic guest displaces the methyl red group from the cavity, a colour change to red is observed. It should be possible to exploit the molecular recognition capability of cyclodextrins to develop a range of such ‘molecular indicators’.

Cyclodextrins are cyclic oligosaccharides consisting of six (α), seven (β) or eight (γ) glucose units. They have a doughnut-like shape and can accommodate a variety of organic substances in the cavity in aqueous solution. Because of this property, cyclodextrins have been used as molecular vessels for reactions or as key compounds for enzyme modelling⁸.

We synthesized methyl-red-modified β -cyclodextrin (MR-CD, Fig. 1b) by reacting 6-deoxy-6-amino- β -cyclodextrin with methyl red in dimethylacetamide, using dicyclohexylcarbodiimide as a condensation reagent. The product was recrystallized from methanol and water (9:1 by volume), and identified by ¹H NMR, infrared, ultraviolet and elemental analysis (calculated proportions for C₅₇H₈₄O₃₅N₄·5H₂O were C, 46.40; H, 6.42; N, 3.80; those found were C, 46.21; H, 6.58; N, 3.84). The solubility of MR-CD is poor in pure water, so we used a 10% ethylene glycol aqueous solution of MR-CD (0.03 mM) for all measurements.

Methyl red (Fig. 1a) is known to change colour from yellow to red when pH is lowered in aqueous solution. This colour change involves structural changes of methyl red; the azo form (A) existing in neutral conditions is converted into protonated ammonium (B) or azonium (C) species both existing in tautomeric equilibrium^{9,10}. The A, B and C species of methyl red and other related compounds show absorptions around 280–340, 390–460 and 510–550 nm, respectively, and methyl red at pH 4.0 shows a strong peak around 525 nm, existing predominantly as C rather than as B. We observed, however, that MR-CD shows a predominant absorption band around 450 nm at pH 4.0, indicating that MR-CD is not converted into the C form even in such an acidic medium. Further decreases in pH lead to reduced absorbance near 450 nm and increased intensity of the shoulder around 550 nm, characteristic of C. Intensity ratios of the two bands (I_{550}/I_{450}) measured at decreasing pH are 0.04 (pH = 6.80), 0.08 (2.43), 0.11 (1.99), 0.17 (1.65), 0.24 (1.43), 0.35 (1.23), 0.43 (1.13), 0.59 (1.00) and 0.74 (0.87). Because the absorbance around 320 nm also increases with decreasing pH, the B species must coexist in these acidic solutions. It is therefore obvious that the azo group in the methyl red moiety of MR-CD is protected from protonation even in an acidic medium, being included in the β -cyclodextrin cavity. This result is consistent with data reported for native β -cyclodextrin and some azo dyes¹⁰.

We observed a colour change of MR-CD from yellow to red on addition of some organic compounds. Figure 2 shows absorption spectra of MR-CD, alone and in the presence of 1-adamantanecarboxylic acid as a guest in a 10% ethylene glycol aqueous solution at pH 1.60. Marked spectral changes occur with increasing concentration of 1-adamantanecarboxylic acid, the band around 455 nm decreasing in its intensity while the band around 510 nm becomes a predominant peak. This spectral behaviour demonstrates that MR-CD releases its methyl red moiety from the cavity into the bulk water environment, and protonation occurs at the azo group, resulting in a structural change from A or B to C (Fig. 3). This structural feature was confirmed from the circular dichroism spectra of MR-CD. A band of positive dichroism around 425 nm indicates that the methyl red moiety is included in the cavity with an orientation parallel to the cyclodextrin axis¹¹; the intensity of the band decreases and a new negative band appears around 520 nm on guest addition. This negative band appearing in the absorption region of C suggests that the methyl red moiety is oriented perpendicular to the cyclodextrin axis¹¹ or is displaced along the cyclodextrin axis in the complex¹². MR-CD therefore undergoes a guest-responsive conformational change, relocating the methyl red moiety from the inside of the cavity toward its outside; this

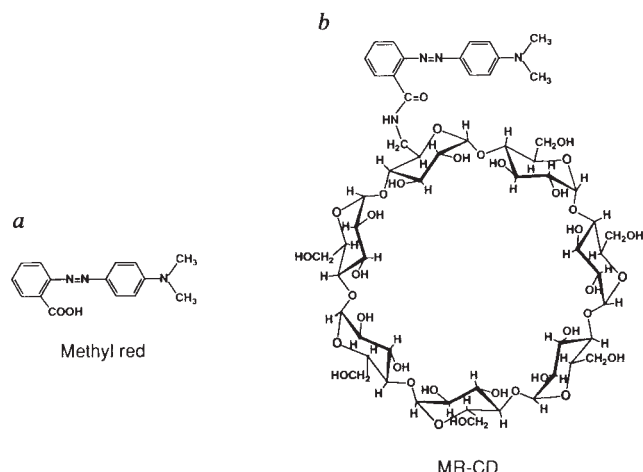


FIG. 1 Structures of methyl red (a) and methyl-red-modified β -cyclodextrin (b).

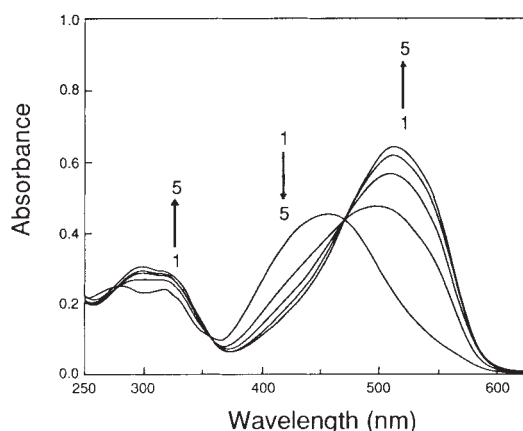
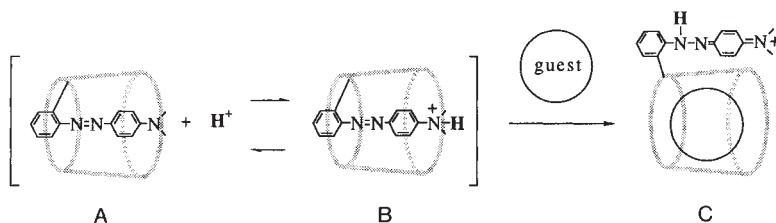


FIG. 2 Absorption spectra of MR-CD (0.03 mM) in a 10% ethylene glycol aqueous solution (pH 1.6) at five concentrations of 1-adamantanecarboxylic acid (1, 0; 2, 0.15; 3, 0.30; 4, 0.45; 5, 0.60 mM). The order of the five traces is indicated.

FIG. 3 Schematic representation of the guest-induced conformational change of MR-CD that causes the colour change.



change of structure is consistent with our work on other modified cyclodextrins^{6,7}.

We have examined the sensor ability of MR-CD using various compounds. L-Borneol (compound **1**), L-camphor (**2-L**), D-camphor (**2-D**), L-fenchone (**3-L**), and D-fenchone (**3-D**) are bicyclic monoterpenes; L-menthol (**4-L**) and D-menthol (**4-D**) are monocyclic. Geraniol (**5**) and nerol (**6**) are also monoterpenes with *trans* and *cis* forms, respectively. Cyclohexanol (**7**), cyclooctanol (**8**), 1-adamantanol (**9**) and 1-adamantanecarboxylic acid (**10**) were used to test the effect of guests of different sizes. The increase of absorbance at 510 nm relative to its original value ($\Delta I/I^0$) was used as a sensitivity parameter. Results obtained at a guest concentration of 0.3 mM are shown in Fig. 4. Of the 13 guests investigated here, adamantane derivatives **9** and **10** showed the highest degrees of sensitivity. For enantiomers **2**, **3** and **4**, sensitivity to the L-isomer was higher than to the D-isomer (1.48-, 3.20- and 1.18-fold preference, respectively). This means that the chirality of D-glucose residues forming the cyclodextrin wall of MR-CD enables MR-CD to exhibit chiral discrimination for optical isomers. The open chain monoterpenes **5** and **6** were detected with 1.7-fold preference for the *trans* form. The final order of sensitivities was $10 > 9 > 1 > 8 > 2-L > 4-L > 2-D \geq 4-D > 3-L > 5 > 3-D > 6 > 7$. To examine the correlation between sensitivity and binding constants in this system, we obtained the binding constants of MR-CD for **1**, **7**, **8**, **9** and **10** from the guest-induced absorption variations. The values were in the order 10 (binding constant $4,890 \text{ M}^{-1}$) > 9 (709 M^{-1}) > 1 (231 M^{-1}) > 8 (35 M^{-1}) > 7 (17 M^{-1}), which coincides with the order of the sensitivity values, and consequently the binding strength of MR-CD for each guest may be considered to be reflected in the sensitivity value. The minimum concentrations detectable by absorption changes under the present conditions were 7.5×10^{-6} , 4.4×10^{-5} , and $7.9 \times 10^{-5} \text{ M}$ for **10**, **9**, and **1**, respectively.

The results suggest that a range of organic compounds could be detected by colour changes on this basis. There are many azo dyes of different sizes and shapes that can be connected to cyclodextrins, and development of colour-changeable

sensors each with unique molecular recognition ability is now under way. □

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1. Löhr, H. G. & Vögtle, F. *Acc. chem. Res.* **18**, 65–72 (1985).
2. Cram, D. J., Carmack, R. A. & Helgeson, R. C. *J. Am. chem. Soc.* **110**, 571–577 (1988).
3. Kaneda, T., Umeda, S., Ishizaki, Y., Kuo, H. & Misumi, S. *J. Am. chem. Soc.* **111**, 1881–1883 (1989).
4. Ueno, A., Suzuki, I. & Osa, T. *J. Am. chem. Soc.* **111**, 6391–6397 (1989).
5. Ueno, A., Suzuki, I. & Osa, T. *Analyt. Chem.* **62**, 2461–2466 (1990).
6. Ueno, A. *et al. Chem. Lett.* 605–608 (1990).
7. Minato, S., Osa, T. & Ueno, A. *J. chem. Soc. Chem. Commun.* 107–108 (1991).
8. Bender, M. L. & Komiyama, M. *Cyclodextrin Chemistry* (Springer, Berlin, 1978).
9. Yamamoto, S., Nishimura, N. & Hasegawa, S. *Bull. chem. Soc. Jpn* **46**, 194–198 (1973).
10. Buvari, A. & Barcza, L. *J. Incl. Phenom.* **7**, 313–320 (1989).
11. Harata, K. & Uedaira, H. *Bull. chem. Soc. Jpn* **48**, 375–379 (1975).
12. Kodaka, M. & Fukaya, T. *Bull. chem. Soc. Jpn* **62**, 1154–1157 (1989).

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Evidence for a decline of PCBs and PAHs in rural vegetation and air in the United Kingdom

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RELIABLE data on persistent organic contaminants in the environment are needed to evaluate strategies to limit their dispersal. Long-term data are often not available, however, because the chemicals in question were not routinely analysed in the past. Although attempts have been made to assess temporal trends by analysis of environmental samples deposited in discrete or identifiable layers (in sediment or peat cores)^{1–3}, these media may be disturbed *in situ* or give poor temporal resolution, or the contaminants may be subject to post-depositional changes. Polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) are persistent and toxic^{4–9} contaminants for which no long-term global ambient monitoring data exist. Plant foliage is a reliable monitor of ambient levels of vapour-phase compounds in air^{10–16} and here we present an analysis of archived herbage samples (1965–89) which shows that air concentrations of lower chlorinated PCBs in rural England have decreased by up to a factor of 50 between 1965–69 and 1985–89. High-molecular-weight PCBs and PAHs have also decreased in concentration, but not to such a great extent.

PCBs, first manufactured in 1929, were not reported in environmental samples until 1966. Their industrial use has been restricted in Europe and North America since the 1970s^{2,8}. Even today, however, PCB concentrations in biota are high enough to be implicated in causing adverse reproductive effects in marine mammals and birds^{6,8,9}. PAHs, many of which are carcinogens and/or mutagens, are the product of incomplete combustion, notably of fossil fuels, so environmental levels are strongly influenced by anthropogenic activities¹⁷. Low-molecular-weight PAHs exist primarily in the vapour phase in air, whereas multi-ringed species predominate in the particulate

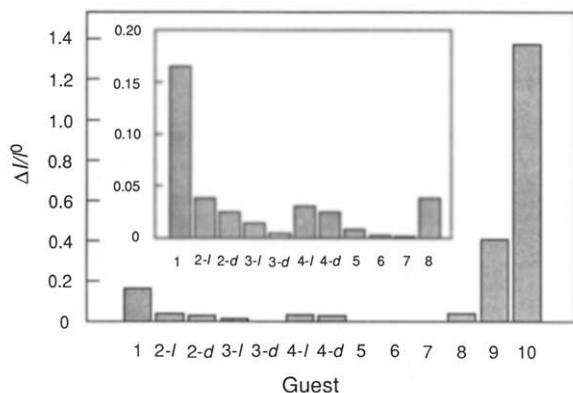


FIG. 4 Sensitivity factors of MR-CD (0.03 mM) in a 10% ethylene glycol aqueous solution for various guests (0.3 mM) as given by the changes in absorbance at 510 nm. Inset: data for all compounds except for **9** and **10** at an enlarged sensitivity scale.

phase (see Table 1). Concentrations of the latter will therefore presumably be more strongly influenced by reductions in the use of coal¹⁷, a shift away from inefficient domestic coal burning¹⁷, and gradual improvements in air quality due to the adoption of emission control technologies. PCBs partition between the vapour phase and inert lipophilic materials, such as the waxy cuticle of leaves. They are strongly sorbed and recalcitrant in soils^{17,19}. Both PCBs and PAHs are essentially unavailable for uptake by plant roots²⁰. Deposition on the foliar portion of plants is a function of air concentrations, although the precise quantitative relationship between air and foliage concentrations is not fully defined¹⁰⁻¹⁴.

Reports of temporal trends for PCBs and PAHs from sediment cores in remote or rural areas indicate how baseline values have changed over periods of decades or centuries¹. These studies have rather poor temporal resolution, however, and may not accurately reflect ambient conditions if the compounds are recycled in the water column or sediments¹⁻³.

Here we report part of an ongoing programme which has used archived soil and vegetation samples from the long-term experimental plots at Rothamsted to investigate pollutant trends in agroecosystems^{17,19-22}. The experimental plots were originally established to identify the effects of nutrients on crop yield. The 1-year grass-clover ley from which the herbage samples were taken was sown and established in autumn. The crop was cut at the silage stage and three harvests were normally taken each year. Samples had been stored at Rothamsted in sealed, air-tight metal containers after being air-dried and milled. Changes in compound composition during sample processing and storage are therefore unlikely²². We prepared annual composite samples by bulking individual harvests in proportion to yield; the yearly samples were then coded and analysed 'blind'. Crop yields fluctuated between 5,200 and 16,000 kg dry mass per hectare, which is typical of lowland regions of the United Kingdom. Samples (12 g) were Soxhlet-extracted with dichloromethane (DCM) and split either for analysis of PCBs or PAHs. The fraction for PCB analysis was transferred to hexane and cleaned using 10 g of 1.25% deactivated Florisil. PCB congeners 30 and 209 were used as recovery and retention standards. More than 40 PCB congeners were originally screened by capillary gas chromatography with electron capture and mass-selective detection^{23,24}. Twenty-five congeners across the full range of isomers were then selected to derive the Σ (total) PCB concentrations. The fraction for PAH analysis was retained in DCM and cleaned on Florisil and with Millex filters before analysis by gradient high-performance liquid chromatography with fluorescence detection. Thirteen compounds, varying between the two-ringed species naphthalene and the seven-ringed coronene, were quantified¹⁹.

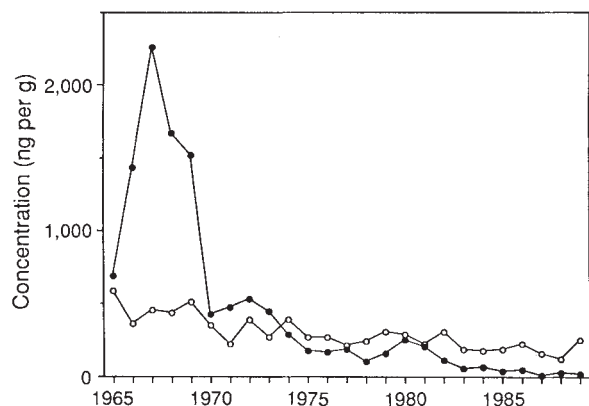


FIG. 1 Temporal trends in the PCB and PAH content of Rothamsted herbage (solid circles, Σ PCB; open circles, Σ PAH).

TABLE 1 Selected PCB congeners and PAH compounds in UK urban air

Compound	Percentage in the vapour phase	Contribution to total PCB/PAH content of air (%)
PCBs*		
18	96	20
28	95	10
52	97	10
118	93	1.7
138	79	2.2
153	84	2.8
183	85	0.7
PAHs		
Phenanthrene	99	47
Anthracene	97	3
Fluoranthene	83	14
Pyrene	87	9
Benzo[<i>b+k</i>]fluoranthene	3	2
Benzo[<i>a</i>]pyrene	<1	1
Benzo[<i>ghi</i>]perylene	2	2

Averaged weekly data from inner-city Manchester, United Kingdom for the first 6 months of 1991, obtained with Hi-Vol air samplers using glass-fibre filters for the particulate phase and polyurethane foam plugs for trapping vapour phase compounds.

* IUPAC numbering system is used. Note: sampling artefacts may bias results on the partitioning of these compounds between vapour and particulate phases, enhancing the estimate of the proportion in the vapour phase (see ref. 18). This is unlikely to have affected the general distribution greatly.

Annual herbage PCB concentrations have declined substantially since the late 1960s (Fig. 1). Data averaged for 5-year intervals (Table 2) show that the Σ PCBs in the samples from the late 1980s were just 2% (28 ng Σ PCB per g dry weight) of the concentration between 1965 and 1969 (1,510 ng per g). Concentrations declined appreciably during the early 1970s and then more slowly throughout the remainder of the study period. Concentrations have been consistently low since about 1982, at <60 ng per g. The Σ PCB burden of all the samples was dominated by the less-chlorinated species, particularly congeners 15 (dichlorinated), 18 and 28 (both trichlorinated). The two trichlorinated compounds always made up >40% of the Σ PCB load (Table 2). These congeners also dominate the mixture of PCBs in air sampled in the United Kingdom, with >90% present in the vapour phase (Table 1). Other di-, tri- and tetrachlorinated species (namely 8, 37, 40, 44, 52, 61/74 and 66) each made up 1–5% of the Σ PCB, whereas the highly chlorinated compounds were only minor herbage components. This differs markedly from the mixture of PCBs in human tissues²⁴ and biota^{8,9}, which is dominated by highly chlorinated species (such as congeners 138, 153, 170, 180). Interestingly, there has been a gradual shift in the relative proportions of the herbage components through time. Table 2 shows that the di-, tri- and tetrachlorinated congeners are present in the most recent samples (1985–89) at only ~2% of the peak 1965–69 levels, whereas pentachlorinated congeners and above (for example, 118, 138, 153 and 183) have declined to ~10–16% of their peak values. A similar change has been reported⁸ for long-term PCB trends in aquatic-feeding birds from North America, and the congener mix in those samples was also dominated by PCBs with five or more chlorine atoms. This shift in the dominance of congeners reflects the greater environmental persistence of the heavier compounds^{8,9,19}. It will be accentuated by bioaccumulation processes⁸ and will introduce systematic errors in the quantification of 'total PCBs' by the packed-column gas chromatography techniques used in some long-term biological monitoring programmes²⁵.

The very high PCB concentrations noted in the samples for 1966–69 are particularly interesting. The mixture of congeners in these samples was the same as that in other samples from the early years, implying that local uncontrolled releases of

TABLE 2 Concentrations of PCB congeners and total PCB

Years	15 (2)	18 (3)	28 (3)	40 (4)	PCB congeners*		138 (6)	153 (6)	183 (7)	ΣPCB†
					52 (4)	118 (5)				
1965–69	162 (100)‡	280 (100)	401 (100)	30 (100)	117 (100)	2.4 (100)	1.25 (100)	1.12 (100)	0.42 (100)	1,510 (100)
1970–74	46 (28)‡	72 (26)	117 (29)	8.2 (27)	34 (29)	1.3 (54)	0.66 (53)	0.49 (44)	0.15 (36)	435 (29)
1975–79	17 (10.5)‡	26 (9.3)	42 (10.5)	3.5 (11.7)	13 (11.1)	0.62 (26)	0.28 (22)	<0.15 (<14)	0.13 (31)	156 (10.3)
1980–84	12 (7.4)‡	18 (6.4)	37 (9.2)	3.2 (10.7)	12 (10.3)	0.84 (35)	0.42 (34)	<0.15 (<14)	0.09 (22)	136 (9.0)
1985–89	4.0 (2.5)‡	5.5 (2.0)	8.9 (2.2)	0.79 (2.6)	2.8 (2.4)	0.23 (9.6)	<0.1 (<8)	<0.15 (<14)	0.07 (16)	28 (1.8)

Selected PCB congeners and ΣPCB concentrations (ng per g dry weight) for grouped 5-year intervals from the Reference Plots at Rothamsted (figures in brackets are percentages of the individual congener concentrations relative to their peak values in 1965–69).

* IUPAC congener numbers are given, with the number of chlorine atoms shown in parentheses.

† ΣPCB is the sum of IUPAC congeners 6, 8, 10, 15, 18, 28, 37, 40, 44, 52, 54, 61/74§, 66, 77/110§, 82/151§, 99, 101, 105, 118, 138, 149, 153, 183, 187. § marks coeluting pairs of congeners not quantified separately by gas chromatography/electron capture detection.

‡ For example, the averaged concentration for the 1970–74 time interval was 28% of that for the 1965–69 interval. By 1985–89 the averaged concentration had diminished to 2.5% of that for the 1965–69 interval.

TABLE 3 Selected PAH compounds and ΣPAH concentrations for grouped 5-year intervals

Years	Phen	Anth	Fluor	Pyrene	B[b+k]F	B[a]P	B[ghi]P	ΣPAH
1965–69	201 (100)†	1.32 (100)	31 (100)	45 (100)	29.3 (100)	2.1 (100)	60 (100)	472 (100)
1970–74	128 (64)	0.92 (70)	16 (52)	22 (49)	25.2 (86)	1.9 (90)	49 (82)	247 (52)
1975–79	86 (43)	0.50 (38)	16 (52)	20 (44)	19.0 (65)	1.6 (76)	44 (73)	261 (55)
1980–84	71 (35)	0.52 (39)	15 (48)	19 (42)	11.3 (39)	1.7 (81)	42 (70)	239 (51)
1985–89	43 (21)	0.52 (39)	9.0 (29)	18 (40)	7.9 (27)	2.1 (100)	37 (62)	187 (40)

Concentrations are in ng per g dry weight.

* ΣPAH is the sum of compounds: Naphthalene; acenaphthene/fluorene‡; phenanthrene (Phen); anthracene (Anth); fluoranthene (Fluor); pyrene; benz[a]anthracene/chrysene‡; benzo[b+k]fluoranthene (B[b+k]F); benzo[a]pyrene (B[a]P); benzo[ghi]perylene (B[ghi]P); coronene. ‡ indicates coeluting compounds.

† See Table 2 for explanation of figures in parentheses.

PCBs were probably not responsible. The high levels observed are generally consistent with peat-core data from the mid-latitudes of eastern North America, which Rapaport and Eisenreich² interpreted as indicating that levels in the late 1960s were higher than in the 1970s.

The Rothamsted trends suggest that primary sources of PCBs to the environment have been reduced substantially because their use in 'open' systems has been limited²⁶. In the United Kingdom, the sole manufacturer of PCBs controlled their sale for particular 'open' uses after 1971 (ref. 26), probably influencing environmental releases in the early 1970s. But important residues continue to re-cycle through the United Kingdom environment by deposition and volatilization between air, soil and water bodies, or are slowly escaping to the air from uncontrolled secondary sources (such as landfills).

The reduction in ΣPAH concentrations in vegetation throughout the time series is less marked than that for the PCBs (see Fig. 1). Nonetheless, the improvement is clear (Table 3) and presumably reflects a reduction in emissions from combustion¹⁷. The largest decrease in concentration was in the 5-year period at the start of the 1970s. Phenanthrene, which dominates the ΣPAH burden of urban air in the United Kingdom (Table 1), was also the main constituent in herbage (Table 3). Comparatively little reduction was observed in the higher-molecular-weight compounds which exist in the air principally in the particular phase. This may imply that re-suspension of 'old' PAHs associated with particulate material continues to be an important source of surface contamination on herbage.

The decline of recalcitrant trace organics in herbage (and therefore air) is encouraging. But it should be noted that concentrations of PAHs and polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/Fs) in soils in the United Kingdom (including those at Rothamsted) are generally still increasing^{18,19,22}, as deposition rates generally exceed removal in crops and losses through degradation, volatilization and leaching^{17,21}. It is also interesting to speculate on the longer-term fate of PCBs from the United Kingdom and other countries at temperate latitudes where they have been used most extensively⁹. Although PCBs

can be selectively degraded in soils²⁷ and air²⁸, they are among the more persistent and mobile of trace organics. Tanabe⁹ has argued that the marine environment will continue to be a net sink for terrestrially derived PCBs, through atmospheric deposition. In terrestrial systems, however, it seems likely that (as for hexachlorobenzene and other organochlorines with similar Henry's Law constants and vapour pressures), a proportion of these semivolatile compounds may be transported and deposited by 'cold condensation' processes^{13,29} to Arctic and sub-Arctic regions¹³, where their rate of recycling and degradation will be noticeably slower under the prevailing cooler climatic conditions. □

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1. Historical Monitoring, Monitoring and Assessment Research Centre Tech. Rep. No 31 (University of London, 1985).
2. Rapaport, R. A. & Eisenreich, S. J. *Envir. Sci. Technol.* **22**, 931–941 (1988).
3. Eisenreich, S. J., Capel, P. D., Robbins, J. A. & Bourbonniere, R. *Envir. Sci. Technol.* **23**, 1116–1126 (1989).
4. International Programme on Chemical Safety *Environmental Health Criteria for PCBs and PCTs* (IPCS, Geneva, 1989).
5. International Agency for Research on Cancer *Monog. on the Evaluation of the Carcinogen Risk of Chemicals to Humans*, Vol. 32 (IARC, Lyons, 1983).
6. Reijnders, P. J. H. *Nature* **324**, 456–457 (1986).
7. Atlas, E. L. & Giam, C. S. *Science* **211**, 163–165 (1981).
8. Norstrom, R. J. in *Hazards, Decontamination and Replacement of PCB*, Ch. 5 (ed. Crine, J.-P.) 85 (New York, Plenum, 1988).
9. Tanabe, S. *Envir. Pollut.* **50**, 5–28 (1988).
10. Buckley, E. H. *Science* **216**, 520–522 (1982).
11. Buckley, E. H. *Recent Adv. Phytochem.* **21**, 175–201 (1987).
12. Riederer, M. *Envir. Sci. Technol.* **24**, 829–836 (1990).
13. Calamari, D. et al. *Envir. Sci. Technol.* **25**, 1489–1495 (1991).
14. Eriksson, G., Jensen, S., Kylin, H. & Strachan, W. *Nature* **341**, 42–44 (1989).
15. Manchester-Neesvig, J. B. & Andren, A. W. *Envir. Sci. Technol.* **23**, 1138–1148 (1989).
16. Baker, J. E. & Eisenreich, S. J. *Envir. Sci. Technol.* **24**, 342–352 (1990).
17. Jones, K. C. et al. *Envir. Sci. Technol.* **23**, 95–101 (1989).
18. McDow, S. R. & Huntzicker, J. J. *Atmos. Envir.* **A24**, 2563–2571 (1990).
19. Wild, S. R., Waterhouse, K. S., McGrath, S. P. & Jones, K. C. *Envir. Sci. Technol.* **24**, 1706–1711 (1990).
20. Wild, S. R., Berrow, M. L., McGrath, S. P. & Jones, K. C. *Envir. Pollut.* **75**, (1992).
21. Kjeller, L.-O., Jones, K. C., Rappe, C. & Johnston, A. E. *Envir. Sci. Technol.* **25**, 1619–1627 (1991).
22. Jones, K. C., Grimmer, G., Jacob, J. & Johnston, A. E. *Sci. tot. Envir.* **78**, 117–130 (1989).
23. Jones, K. C. *Sci. Tot. Envir.* **68**, 141–159 (1988).
24. Duarte-Davidson, R., Burnett, V., Waterhouse, K. S. & Jones, K. C. *Chemosphere* **23**, 119–131 (1991).
25. Ministry of Agriculture, Fisheries and Food, *Rep. Working Party on Pesticide Residues (1982–85). Food Surveillance Pap. No 16* (HMSO, London, 1986).

26. Dept of the Environment *Waste Management Pap.* No 6 (HMSO, London, 1976).
 27. Abramowicz, D. A. *Crit. Rev. Biotech.* **10**, 241–251 (1990).
 28. Sedlak, D. L. & Andren, A. W. *Envir. Sci. Technol.* **25**, 1419–1427 (1991).
 29. Mackay, D. & Clark, K. E. in *Organic Contaminants in the Environment*, Ch. 6 (ed. Jones, K. C.) (Elsevier, 1991).

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Biodegradation of metal citrate complexes and implications for toxic-metal mobility

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THE presence of synthetic and naturally occurring chelating agents in nuclear and toxic-metal wastes is a major concern because of their potential to enhance mobilization of metal ions away from the disposal sites^{1–3}. Of particular interest is citric acid, which is present in low-level and transuranic radioactive wastes^{4,5} and in domestic and industrial wastes (as washing fluids, for instance), as well as being found naturally. Citrate ions form multidentate, stable complexes with a variety of toxic metals and radionuclides; but biodegradation of these complexes, precipitating the metal ions as insoluble hydroxides, oxides or other salts, may retard migration. Here we report a study of the biodegradation of citrate complexes of Ca, Fe(II), Fe(III), Cd, Cu, Ni, Pb and U. Several of these complexes were not readily degraded by bacteria, and the biodegradability depended on the chemical nature of the complex, not on the toxicity of the metal to the bacteria. This resistance to biodegradation implies that citrate complexation may play an important part in migration of these hazardous wastes.

Mixed cultures of sewage bacteria and axenic cultures of *Pseudomonas* sp. and *P. pseudoalcaligenes* have been found⁶ to metabolize metal citrate complexes of Ca, Fe, Al and Mg at different rates and extents. *Klebsiella* sp., also isolated from sewage, degrades magnesium citrate but fails to degrade Cd, Cu, or Zn citrate complexes⁷. These studies showed that the lack of biodegradation was not due to toxicity of the metal. The nature and biodegradability of the complexes formed between the metal and citric acid were not, however, investigated.

Citric acid forms mononuclear, binuclear or polynuclear complexes depending on the type of metal (Fig. 1). The types of complexes formed between equimolar amounts of citric acid and Ca^{2+} , Cd^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Ni^{2+} , Pb^{2+} or UO_2^{2+} were

FIG. 1 Types of metal citrate complexes. a, Citric acid; b, bidentate complex involving two carboxylic acid groups; c, tridentate complex involving two carboxylic acids and the hydroxyl group; d, binuclear complex with UO_2^{2+} .

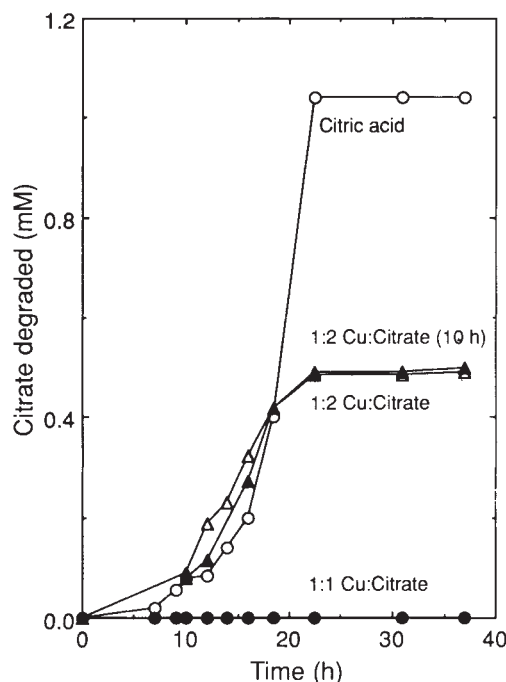
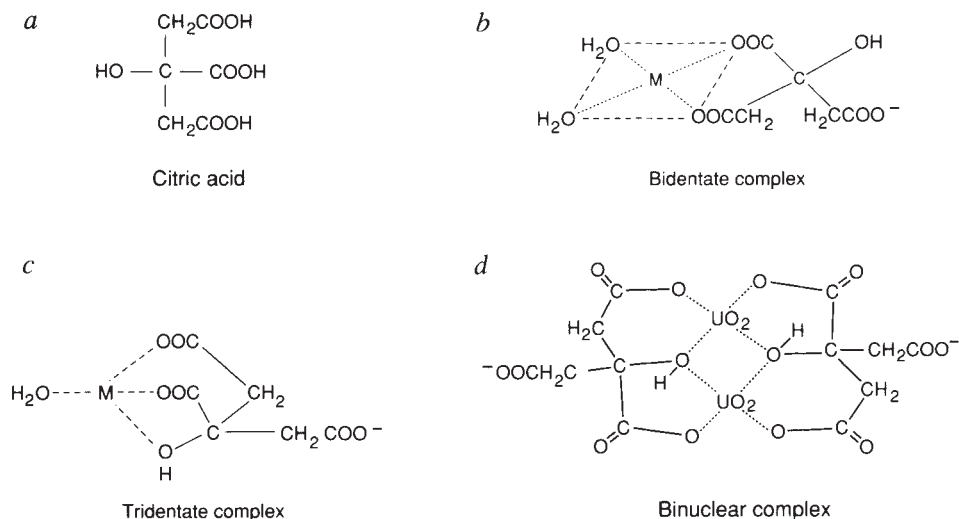


FIG. 2 Degradation of citrate in the presence of tridentate copper citrate complex. A 1:1 copper citrate complex is not biodegraded. The excess free citrate (0.52 mM) in a 1:2 copper citrate complex is completely degraded, indicating that the complex is not toxic.

found by potentiometric titrations using a 1:1 metal citrate complex (containing 0.52 mM of metal and of citric acid). Additional information on the nature of the complexes was obtained from published data. Calcium, ferric iron and nickel formed bidentate, mononuclear complexes with two carboxylic acid groups of the citric acid molecule^{8–13}. Copper, ferrous iron, cadmium and lead formed tridentate, mononuclear complexes with citric acid involving two carboxylic acid groups and the hydroxyl group^{8,14–16}. Uranium formed a binuclear complex with two uranyl ions and two citric acid molecules involving four carboxylic acid groups and two hydroxyl groups^{17–19}.

We isolated a citrate-metabolizing bacterium, *P. fluorescens* (ATCC no. 55241), from the leachate sample collected from the low-level radioactive waste disposal site, West Valley, New York. The bacterium degraded citrate at a maximum rate of $57 \mu\text{mol h}^{-1}$. To investigate its ability to degrade several metal

TABLE 1 Biodegradation of 1:1 metal citrate complexes.

Type of complex	Metal	Formula	Formation constant (log K)	Biodegradation rate ($\mu\text{mol h}^{-1}$)	(%)	Reference
Bidentate	Calcium	(Ca cit) ⁻	3.5	77 $\pm$ 10*	100	6, 8
	Nickel	(Ni cit) ⁻	5.4	9 $\pm$ 1	70	12, 13
	Fe ³⁺	(Fe(OH) ₂ cit) ²⁻	1.9-2.6	16 $\pm$ 1	100	6, 11
Tridentate	Fe ³⁺	(Fe(OH) cit) ⁻	9.4	1 $\pm$ 0	16	6, 9
	Fe ²⁺	(Fe cit) ⁻	4.4	ND	0	9, 14
	Copper	(Cu cit) ²⁻	5.9	ND	0	7, 8
	Cadmium	(Cd cit) ⁻	3.7	ND	0	7, 15
	Lead	(Pb cit) ⁻	4.4	ND	0	16
Binuclear	Uranium	((UO ₂) ₂ cit) ²⁻	7.4	ND	0	18

ND, none detected.

* Values are mean $\pm$ 1 standard error of the mean (SEM).

citrate complexes we grew the bacterium in a defined medium developed in accordance with equilibrium calculations so that the metal citrate complex was the predominant species⁶. The phosphate buffer and inorganic phosphate in the medium were replaced with PIPES buffer and glycerolphosphate to prevent the precipitation of metals. The pH was adjusted to 6.1 and the culture was incubated in triplicate at 26 °C. Citric acid was analysed by high-pressure liquid chromatography, metals by atomic absorption spectroscopy and uranium by spectrophotometry or by inductively coupled plasma mass spectrometry.

The results in Table 1 show that the bidentate complexes of Ca, Fe(III) and Ni were readily degraded, with calcium citrate showing the fastest rate. Ferric citrate was completely degraded but at a slower rate. The iron that was released formed a hydroxide precipitate. In contrast, nickel citrate complex showed only 70% degradation. During the degradation of this complex the pH of the culture medium increased to 7.6, causing changes in the complex structure. At acid to neutral pH the complex is present as bidentate (Ni cit)⁻. At alkaline pH, however, the hydroxyl group was ionized, forming a tridentate complex^{8,20}, and was resistant to further biodegradation.

The tridentate complexes of Cd, Cu and Pb, but not that of Fe(II), were completely resistant to degradation. Ferrous iron initially formed a tridentate complex (Fe cit)⁻. Oxidation and hydrolysis of the ferrous iron resulted in the formation of a tridentate ferric complex (FeOH cit)⁻, which was further hydrolysed to a bidentate complex (Fe(OH)₂ cit)²⁻. The rate of degradation of the ferrous complex depended on the rate of conversion to the more hydrolysed form of the ferric complex. The presence of bacteria accelerated this conversion.

Although the copper citrate complex was not biodegraded (Fig. 2), adding 1:1 copper citrate complex to the culture medium, which contained 0.52 mM of uncomplexed citrate, at the start of the experiment and after 10 h affected neither the growth nor the rate and extent of degradation of excess free citrate. The lack of degradation of the copper citrate complex was not due to its toxicity. Cadmium- and lead-citrate complexes were not biodegraded, but they have been shown to form polynuclear complexes at higher pH with lead citrate precipitating out of solution^{15,16,21,22}. In the presence of excess citric acid, these metals also formed 1:2 mononuclear biligand complexes such as (Cd cit₂)⁴⁻. Glucose added to the growth medium was readily metabolized by the bacteria, suggesting that these

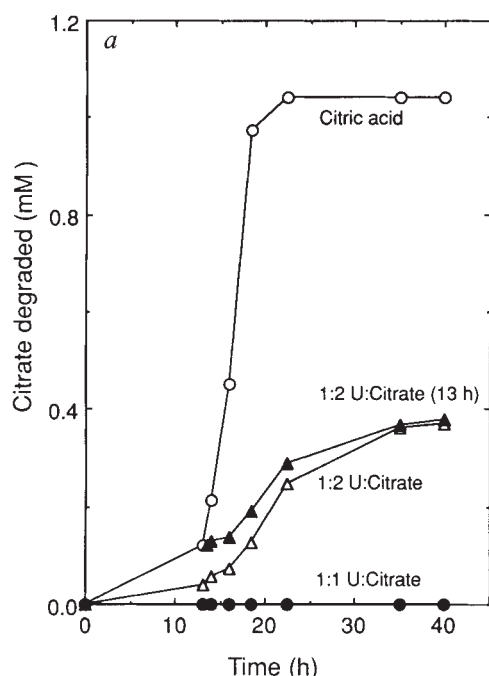
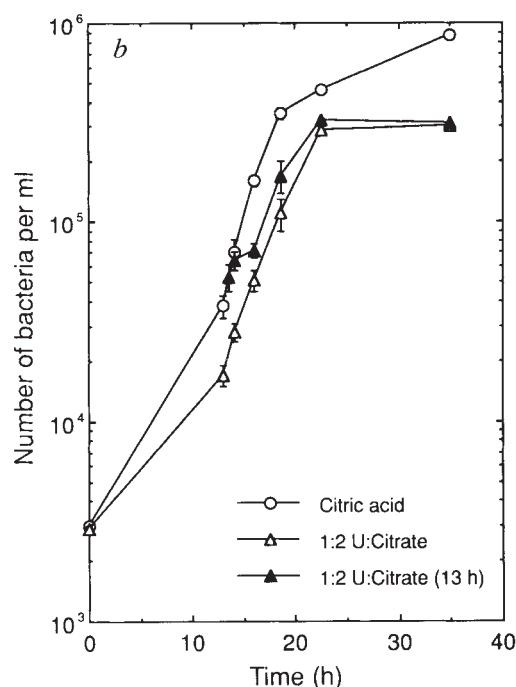


FIG. 3 a, Degradation of citrate in the presence of uranium citrate. The 1:1 complex was not biodegraded. Incomplete degradation in the presence of excess free citrate is due to the formation of a 2:3 uranium citrate complex.



b, Increase in bacterial cell number is observed because the available free citrate is metabolized. The uranium citrate complex is not toxic to bacteria. Error bars are $\pm$ 1 standard error on the mean.

complexes too are non-toxic.

Uranium formed a binuclear complex $((\text{UO}_2)_2 \text{cit}_2)^{2-}$ and was not degraded by the bacteria (Fig. 3a). In the presence of excess citrate, ~35% of the citrate was metabolized regardless of whether the 1:1 uranium citrate complex was added after 0 h or 10 h of growth. The number of bacteria increased, indicating the absence of toxicity, but growth was limited by the presence of uncomplexed citrate (Fig. 3b). It seems that U formed a polynuclear 2:3 complex in the presence of excess citrate, thereby limiting the amount of free citrate available for biodegradation²³. Further experiments with varying uranium: citrate ratios also confirmed this observation.

Several other bacterial cultures isolated from wastes and leachates containing toxic metals and radionuclides degraded citrate complexes of Ca, Fe(III) and Ni, but not those of Cd, Cu, Pb and U. On the basis of the formation constant ($\log K$) of the metal citrate complexes (Table 1), biodegradation should follow the order $\text{Fe(III)(OH)}_2 > \text{Ca} > \text{Cd} > \text{Pb} = \text{Fe(II)} > \text{Ni} > \text{Cu} > \text{U(VI)} > \text{Fe(III)OH}$. Instead we observed the following order: $\text{Ca} > \text{Fe(III)(OH)}_2 > \text{Ni} > \text{Fe(III)OH}$, and the complexes Fe(II) , Cu, Cd, Pb, and U(VI) were not degraded.

These results show that the type of complex formed between the metal and citric acid is important in determining its biodegradability. The presence of a free hydroxyl group of the citric acid seems to be the key to biodegradation of the metal complex. For example, Ca, Fe(III) and Ni formed mononuclear bidentate complexes and were readily biodegraded, whereas Ca, Cu, Fe(II) and Pb, which formed mononuclear tridentate complexes, and U, which formed a binuclear complex involving the hydroxyl group of the citrate, were not biodegraded. The lack of degradation of tridentate and binuclear complexes was not due to toxicity, but probably limited by the transport and/or

lack of metabolism of the complex by the bacteria^{14,24-26}. In addition to its presence in hazardous and mixed wastes, citric acid can be produced from the degradation of organic constituents of the waste. Citric acid can thus facilitate transport of certain toxic metals and radionuclides from the waste disposal sites by forming stable complexes which are resistant to biodegradation. $\square$

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- Means, J. L., Crerar, D. A. & Duguid, J. O. *Science* **200**, 1477-1481 (1978).
- Francis, A. J. in *Soil Reclamation Processes: Microbiological Analyses and Applications* (eds Tate, R. L. & Klein, D. L.) 279-331 (Marcel Dekker, New York, 1985).
- Francis, A. J. *Experientia* **46**, 840-851 (1990).
- DOE Office of Energy Research *DOE/ER-04921* (US Department of Energy, Washington DC, 1991).
- DOE Office of Energy Research, *DOE/ER-0419* (US Department of Energy, Washington DC, 1989).
- Madsen, E. & Alexander, M. *Appl. envir. Microbiol.* **50**, 342-349 (1985).
- Brynhildsen, L. & Rosswall, T. *Appl. envir. Microbiol.* **55**, 1375-1379 (1989).
- Campi, E., Ostacoli, G., Meirone, M. & Saini, G. *J. inorg. nucl. Chem.* **26**, 553-564 (1964).
- Ham, R. E., Shull, C. M. & Grant, D. M. *J. Am. chem. Soc.* **76**, 2111-2114 (1954).
- Strouse, J., Layten, S. W. & Strouse, C. E. *J. Am. chem. Soc.* **99**, 562-571 (1977).
- Dhar, S. K. & Sihak, S. *J. inorg. nucl. Chem.* **41**, 126-127 (1979).
- Li, N. C., Lindenbaum, A. & White, J. M. *J. inorg. nucl. Chem.* **12**, 122-128 (1959).
- Hedwig, G. R., Liddle, J. R. & Reeves, R. D. *Austr. J. Chem.* **33**, 1685-1693 (1980).
- Villafraña, J. J. & Mildvan, A. S. *J. biol. Chem.* **247**, 3454-3463 (1972).
- Capone, S., DeRobertis, A., DeStefano, C. & Sammartano, S. *Talanta* **33**, 763-767 (1986).
- Bkstrom, L. G. & Olin, A. *Chem. Script.* **13**, 10-15 (1978-79).
- Rajan, K. S. & Martell, A. E. *Inorg. Chem.* **4**, 462-469 (1965).
- Nunes, M. T. & Gill, V. M. S. *Inorg. chim. Acta* **129**, 283-287 (1987).
- Sillen, L. G. & Martell, A. E. *Stability Constants of Metal-Ion Complexes*, Spec. Publ. No. 25, Suppl. No. 1. (Chemical Society, London, 1971).
- Still, E. R. & Wikberg, P. *Inorg. chim. Acta* **46**, 153 (1980).
- Grenthe, I., Wikberg, P. & Still, E. R. *Inorg. chim. Acta* **91**, 25-31 (1984).
- Bottari, E. *Ann. Chim.* **65**, 593-607 (1975).
- Heitner, C. & Bobtelsky, M. *Bull. Soc. Chim. France* **356**, 23 (1954).
- Bergsma, J. & Konings, W. N. *Eur. J. Biochem.* **134**, 151-156 (1983).
- Glusker, J. P. *Acc. chem. Res.* **13**, 345-352 (1980).
- Willecke, K., Gries, E. M. & Oehr, P. *J. biol. Chem.* **248**, 807-814 (1973).

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A shear-strain anomaly following the Loma Prieta earthquake

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BOREHOLE tensor strain instruments deployed along the San Andreas fault for the past ten years have provided sufficient resolution and stability to sample regional tectonic processes, potentially enhancing earthquake prediction capability. Data obtained from the instrument at San Juan Bautista, in the near-field region of the 17 October 1989 Loma Prieta earthquake ($M_s = 7.1$) provide the first opportunity to observe shear strain processes associated with a large earthquake. We previously reported¹ a prominent shear-strain anomaly in those data for more than a year before the earthquake. This anomaly ceased immediately after the earthquake, but, as we report here, a new and higher rate of fault-parallel shear accumulation (2 microstrain per year) was established about four months later and has continued to the present. Associated changes in creep rate are apparent at a number of sites on the surface trace of the fault within 30 km of the strain meter. We propose that the observed strain accumulation results from increased slip around a nearby locked section of the fault, this slip arising from loading by the failed Loma Prieta source region to the north. This model is consistent with suggestions of an increased probability of a moderate earthquake near San Juan Bautista^{9,10}, and with evidence¹² that interactions between fault regions are important in earthquake processes.

A Gladwin borehole tensor strain meter² installed near the San Andreas fault at San Juan Bautista in late 1983 has provided continuous areal and shear strain data with sub-nanostrain ($\text{n}\epsilon$)

resolution and long-term stability better than 100 $\text{n}\epsilon$ per year³. Raw data from the instrument consist of diameter changes in three directions at 120° to each other in the horizontal plane. These are reduced to areal strain ϵ_a , and engineering shear strains γ_1 and γ_2 (roughly parallel to and at 45° to the fault, respectively). The strain meter is grouted into the surrounding rock, and this instrument inclusion is softer to shear than to compression. Observed strain components are therefore scaled by hole coupling parameters⁴ determined by tidal calibration.

Data from borehole inclusions are initially dominated by grout compression of the instrument, by thermally controlled decay as the instrument site re-establishes equilibrium with its surroundings and by an exponential recovery of the virgin stress field relieved at the borehole during the drilling process^{5,6}. The exponential signals have no relevance to the monitoring of regional strain changes and were removed by an exponential least-squares fit over the interval January 1984 to February 1988 (ref. 1). These residuals were then reduced to the strains ϵ_a , γ_1 and γ_2 shown in Fig. 1a.

The dominant signals present are the coseismic strain steps of the Loma Prieta earthquake which in Fig. 1b are removed from the data to make long-term trends more apparent. As previously reported, an anomalous change in γ_1 is apparent by late 1988, showing a remarkably linear strain accumulation of 1 $\mu\epsilon$ per year¹. A smaller change of ~0.3 $\mu\epsilon$ per year was also evident from mid-1988. The azimuth of maximum shear for the accumulating shear strain was roughly parallel to the local San Andreas strike.

Immediately after the earthquake, the strain rate returned for ~10 days to its value before the anomaly, then decreased for two months. By May 1990, following the Chittenden aftershock sequence, the present linear shear accumulation rate of ~2 $\mu\epsilon$ per year with the original sense had been established. During the whole of the period since mid-1988, medium-term changes in ϵ_a and γ_2 have been less than 250 $\text{n}\epsilon$ per year. The strain data indicate that by May 1990, the accumulating strain was

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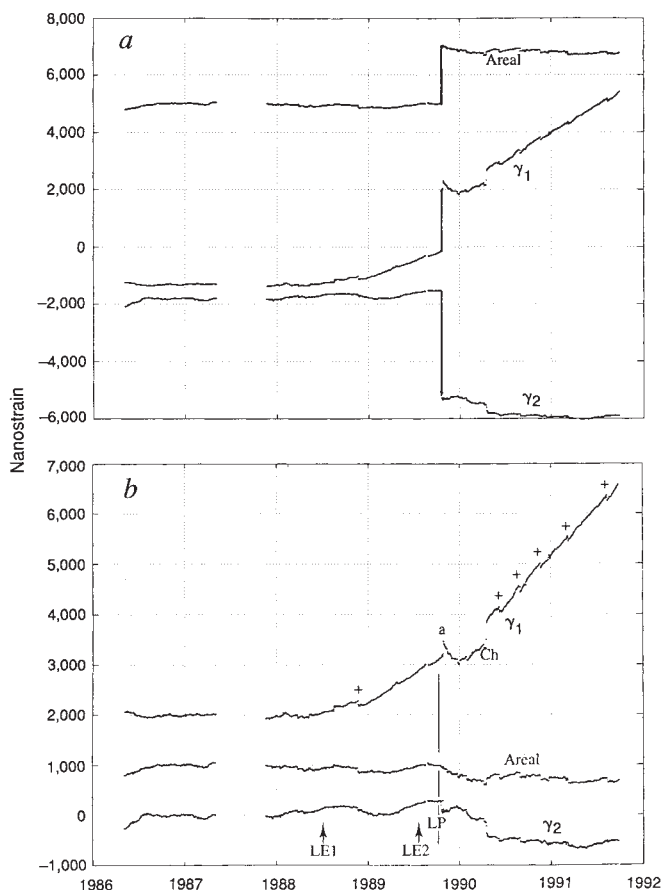


FIG. 1 *a*, Areal strain and shear strains from the SJT borehole tensor strain meter at San Juan Bautista near the San Andreas fault in northern California. Exponential trends have been removed. The dominant feature is the coseismic strain step from the Loma Prieta earthquake. *b*, Removal of this step reveals the details of the strain records, in particular the striking anomaly in the γ_1 component, the relative constancy of the other two strain components and trend reversal on γ_1 for three months following the Loma Prieta earthquake. All steps in the data can be associated with seismic events or nearby creep events: for example, the times of the two Lake Elsmar earthquakes are indicated (LE1, LE2), as are the times of the Loma Prieta earthquake (LP), a Loma Prieta aftershock (A), the Chittenden earthquake sequence (Ch) and creep events also monitored on a nearby creep meter (+).

predominantly shear strain with a maximum azimuth of 318° , close to the local San Andreas fault strike of 310° .

To verify the stability of the instrument and coupling, we investigated the response to earth strain tides using the dominant, thermally uncontaminated tidal components O_1 and M_2 . Results are shown in Fig. 2 for the ϵ_a and γ_1 data sets. It is clear that there have been no significant or systematic changes of tidal admittance on this instrument over the whole period under discussion. In particular, the admittance anomalies before the earthquake suggested for Searle Road dilatometer data⁷ are absent in these tensor strain observations.

Several explanations for the strain anomaly following the Loma Prieta earthquake need to be examined. A non-tectonic source from the instrument or its immediate vicinity is unlikely because of the stability of the tidal response, consistency of our internal instrument checks, stability of the areal strain record and detailed correspondence in time of all observed strain steps with either earthquakes or creep events on nearby creep meters.

Although the strain signals could arise from small-scale processes in a nearby section of the fault, observations of anomalous creep at three sites up to 30 km away indicate a more extended source. Figure 3 shows long-term data from creep meters for 5 sites covering 40 km of the fault south of San Juan Bautista, with long-term trends from ref. 8. A distinct increase in creep rate following the Loma Prieta earthquake is evident on sites XSJ, XHR and CWC, spanning 16 km of the fault. The XFL site (29 km from XSJ) shows only a marginal increase, and the more remote site XMR (40 km) shows no effect.

The creep anomalies in Fig. 3 are atypical, especially for CWC and XHR, and begin at the time of the Loma earthquake. The creep anomaly at XSJ begins about the time of the establishment of the new shear-strain anomaly at SJT. This strongly suggests that these signals are not just the consequence of normal interactions between fault sections in this creeping section, but are linked to the earthquake. We conclude that the failure of the Loma Prieta source region transferred load to the San Juan Bautista region just to its south, resulting in increased creep rate. The simplest explanation of an increased creep rate is frictional response to the increased shear loading parallel to the fault indicated by the coseismic γ_1 step at San Juan Bautista (see Fig. 1*a*).

But slip through creep does not itself result in linearly increasing elastic strain. We suggest that our linear shear-strain anomaly is best explained by continued aseismic slip around a nearby locked section of the fault, the slip being associated with loading transferred from the Loma Prieta source region, particularly

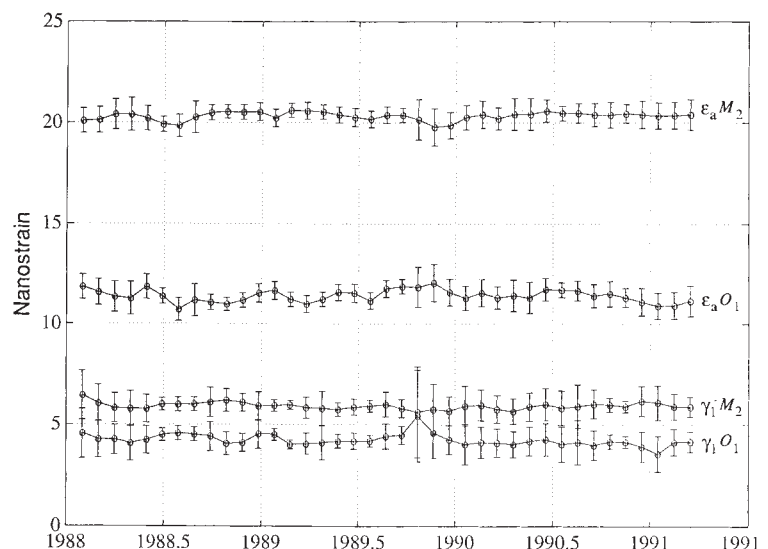


FIG. 2 Amplitude of the M_2 and O_1 tidal components of areal strain ϵ_a and shear strain γ_1 for the period 1988 to the present. Sixty-day windows of 90-min data were used to provide the amplitude of the normalized tidal component every 30 days. The strain step of the Loma Prieta event and other easily identifiable strain steps were removed from the record. Error bars indicate the precision of determination, assuming gaussian noise. There is no significant change in instrument performance for any component.

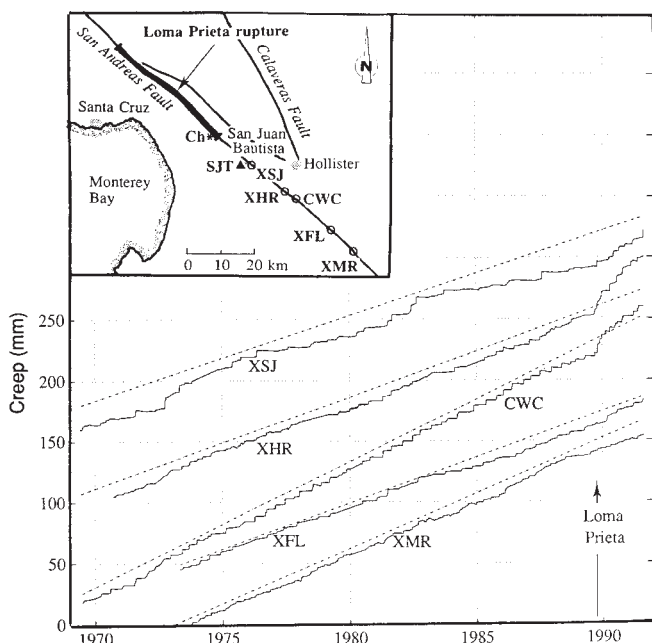


FIG. 3 Surface creep data (provided by K. Breckenridge of United States Geological Survey, Menlo Park) from creep meters along the fault south of San Juan Bautista, with positions of creep meters XSJ, XHR, CWC, XFL and XMR, tensor strain meter SJT, and Chittenden aftershock Ch indicated in the inset map. The trend lines indicated are from Burford⁸. Note that the XMR data have been halved for convenience of plotting.

after the Chittenden aftershock sequence (April 1990). The area near San Juan Bautista is known to experience moderate earthquakes ($M_L \approx 5$) 4–8 km deep, and an earthquake has recently been predicted there after a locked section was identified by seismic quiescence⁹ and creep retardation¹⁰. The shear-strain data are consistent with increased loading on a locked section in this region, and thus an increased probability of a moderate earthquake in the San Juan Bautista region.

We have previously suggested¹ that the strain changes occurring before Loma Prieta were related to a broad regional effect, arguing this from the timing of the Lake Elsman foreshock and a marginal geodetic anomaly¹¹ near the Loma Prieta source. Creep retardation preceding moderate earthquakes in adjacent regions has been observed⁸. The creep data in Fig. 3 also show some evidence for a regional creep anomaly (at XSJ, XHR and CWC) in the two years before Loma Prieta. This provides support, independent of the geodetic data, for our suggestion¹ of a pre-Loma Prieta strain anomaly, at least in the San Juan region. The disappearance of the strain anomaly immediately following the earthquake has further strengthened the case that these anomalous changes were directly associated with the Loma Prieta event. □

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- Gladwin, M. T., Gwyther, R. L., Higbie, J. W. & Hart, R. *Geophys. Res. Lett.* **18**, 1377–1380 (1991).
- Gladwin, M. T. *Rev. scient. Instrum.* **55**, 2011–2016 (1984).
- Gladwin, M. T., Gwyther, R. L., Hart, R. & Francis, M. *J. geophys. Res.* **92**, 7981–7988 (1987).
- Gladwin, M. T. & Hart, R. *Pure appl. Geophys.* **123**, 59–88 (1985).
- Berry, D. S. *Int. J. Rock Mech. Min. Sci.* **4**, 181–187 (1967).
- Berry, D. S. & Fairhurst, C. *Am. Soc. Testing Mat. STP* **402**, 190–206 (1966).
- Johnston, M. J. S. & Linde, A. T. *Eos* **71**, 1461 (1990).
- Burford, R. O. *Pure appl. Geophys.* **126**, 499–529 (1988).
- Wyss, M. & Burford, R. A. *Nature* **329**, 323–325 (1987).
- Sylvester, A. G., Burford, R. O. & Schultz, S. S. *Eos* **71**, 290 (1990).
- Lisowski, M., Prescott, W. H., Savage, J. C. & Svarc, J. L. *Geophys. Res. Lett.* **17**, 1211–1214 (1990).
- Marko, G. M. *J. geophys. Res.* **87**, 7807–7816 (1982).

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Otavipithecus namibiensis, first Miocene hominoid from southern Africa

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WE report here the discovery of a Miocene hominoid from Berg Aukas, Namibia, the first known from the African continent south of equatorial East Africa. This represents a major range extension of Miocene Hominoidea in Africa to latitude 20° S. The holotype, a right mandibular corpus preserving the crowns of the P_4 – M_3 , partial crown and root of the P_3 , partial root of the canine, alveoli for all four incisors, and partial alveolus for the left canine, was found during paleontological explorations of karst-fill breccias in the Otavi region of northern Namibia. The mandible has unique characteristics that differentiate it from other middle Miocene hominoids of Africa and Eurasia and represents the only fossil evidence documenting a pre-australopithecine stage of hominoid evolution in southern Africa. Faunal analyses indicate that the breccia block containing the specimen accumulated during the latter part of the middle Miocene, about 13 ± 1 Myr. Fauna from other breccia blocks at Berg Aukas are of diverse ages, including the earlier part of the middle Miocene, the upper Miocene, Pliocene and Holocene.

Order Primates Linnaeus 1758

Suborder Anthroproidea MIVART 1864

Superfamily Hominoidea GRAY 1825

Otavipithecus gen. nov.

Etymology. *Otavipithecus* Greek meaning ape from Otavi.

Diagnosis. (1) 'Puffy' molar cusps forming distinctive Y5 pattern in which the lingual slope of the hypoconid extends well beyond the midaxis of the tooth (particularly in M_1) and which restrict the size of the trigonid and talonid basins; (2) absence of beaded buccal cingular shelf on molars; (3) presence of protostylar ridges on M_2 and M_3 ; (4) molars 'squared-off', not elongated; (5) $M_2 > M_3 > M_1$; (6) moderate development of the inferior transverse torus; (7) large retromolar space; (8) wear pattern suggestive of thin enamel and/or high dento-enamel relief; (9) M_3 not obscured by the anterior root of the ascending ramus in lateral view; (10) little differential wear on molars; (11) depth of mandible does not markedly decrease mesiodistally; (12) extremely narrow incisor region.

Otavipithecus namibiensis sp. nov.

Etymology. In honour of Namibia, Africa's newest independent nation.

Holotype. BER I, 1'91 (site I at Berg Aukas; first specimen collected in 1991), a right mandible extending from the mesial alveolar wall of the left canine to include the retromolar fossa and the adjacent base of the ascending ramus (but excluding the angle) on the right side and preserving the crowns of P_4 – M_3 ,

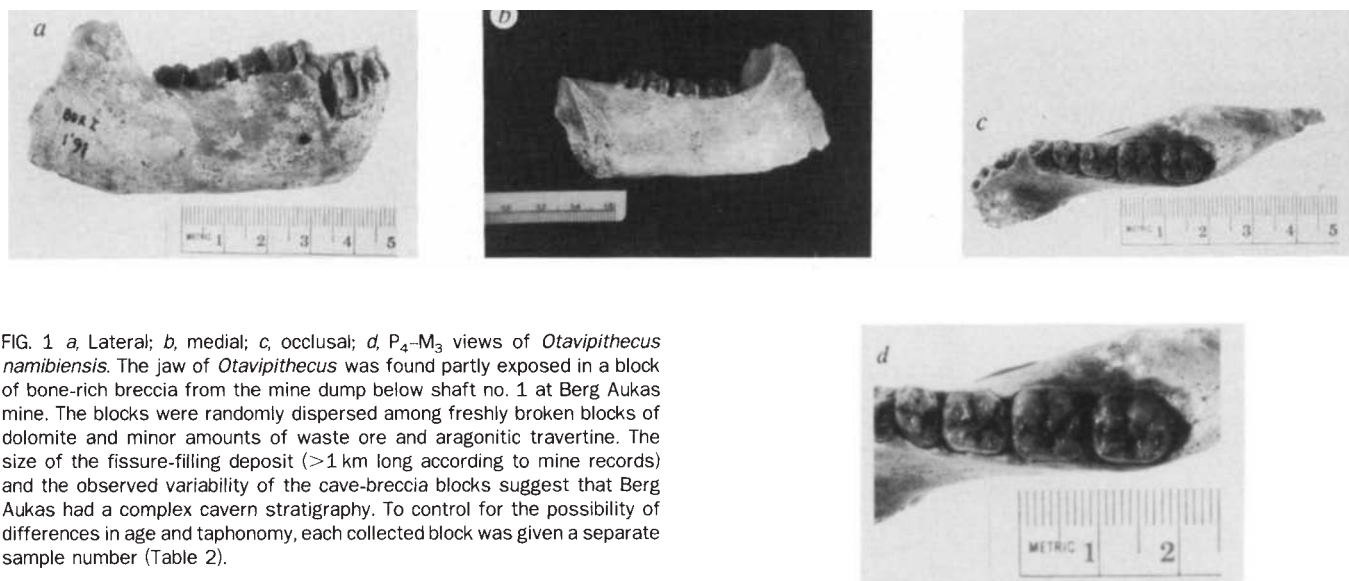


FIG. 1 a, Lateral; b, medial; c, occlusal; d, P₄-M₃ views of *Otavipithecus namibiensis*. The jaw of *Otavipithecus* was found partly exposed in a block of bone-rich breccia from the mine dump below shaft no. 1 at Berg Aukas mine. The blocks were randomly dispersed among freshly broken blocks of dolomite and minor amounts of waste ore and aragonitic travertine. The size of the fissure-filling deposit (>1 km long according to mine records) and the observed variability of the cave-breccia blocks suggest that Berg Aukas had a complex cavern stratigraphy. To control for the possibility of differences in age and taphonomy, each collected block was given a separate sample number (Table 2).

partial crown and root of P₃, partial canine root, and alveoli for all four incisors. The specimen was found by M.P. on 4 June 1991.

Locality. Berg Aukas, Namibia, roughly 20 km east of Grootfontein on the southeastern part of the Otavi Mountains (19° 30' 58" S; 18° 15' 10" E).

Horizon. Fissure filling (breccia) from open cast mine near Shaft No. 1, Berg Aukas.

Age and Distribution. Middle Miocene, known only from type locality.

Diagnosis. Only known species; same as for genus. Measurements are given in Table 1.

The mandibular corpus is characterized by: (1) a mental foramen situated beneath P₃-P₄ at the base of a well developed postcanine fossa; (2) a distinct mylohyoid groove and faint mylohyoid line below which lies a well formed submandibular fossa; (3) an inferior transverse torus that extends slightly more posteriorly than the superior one, ending below the distal end of P₄; and (4) a steeply inclined alveolar plane.

The incisor region is extremely narrow (Table 1). Judging by alveolar size, the lateral incisors were as large, or slightly larger, than the central incisors. Incisor alveoli are roughly 9-10 mm long. Canines were stout teeth (suggesting a male individual) with roots roughly 20 mm long. The large pulp chamber of the right canine is about 2.5 mm in diameter and X-rays reveal that the root apex was still open. There is no diastema between the canine and P₃.

The alveolus for the buccal root of P₃ is about 13 mm long (X-rays and CT reveal a similarly sized lingual root). The P₄ crown has a narrow anterior fovea with a mesio-distally oriented sulcus. Its talonid basin, narrowed by encroachment of a prominent entoconid, extends disto-lingually, thus making the long axis of the tooth oblique to the mesio-distal axis of the molar row. The entoconid is a prominent cusp; the hypoconid, however, is not clearly distinct from the talonid rim. The protoconid and metaconid are bunodont and about equal in height and the plane of the trigonid is not greatly elevated above that of the talonid. The crown lacks the great buccal flare typical of *Sivapithecus* P₄s from Pasalar¹. X-ray shows the two roots of P₄ to be about 11 mm long.

Molars are characterized by puffy, bunodont cusps separated by deep grooves that form a distinctive Y5 pattern with the lingual slope of the hypoconid extending well beyond the mid-axis of the tooth. Anterior and posterior foveas are restricted in size. Tiny crater-like perforations appear on the apices of the

main cusps, a wear pattern characteristic of other hominoids with relatively thin enamel. Enamel thickness over the M₂ protoconid appears to be less than 1 mm thick based on a 1-mm-thin CT scan taken through the cusp (enamel thickness on the broken lingual cusp of P₃ is less than 0.5 mm thick) (see also ref. 2). There is little differential wear on the molars even though this was an adult animal in which the M₃ had already reached the occlusal plane. The M₃ does not taper distally as in *Proconsul* and most *Dryopithecus* species. X-rays reveal large pulp chambers in all the molars, particularly M₃, where the roots appear to have coalesced into a common root with one large pulp chamber. The M₃ would be considered 'mesotaurodont', the M₁ and M₂ cynodont, and the P₄ hypotaurodont³.

Until the discovery of *Otavipithecus namibiensis*, no Miocene hominoid had ever been found south of the equatorial sites in Kenya and Uganda. Most Miocene hominoid sites occur in southern Eurasia between latitudes 23° N and 50° N, extending from Spain in the west to China in the east, and in East Africa (Fig. 2). This discovery is thus a major range extension of the Hominoidea by some 10° of latitude south of the southern limits of extant African apes.

TABLE 1 Measurements of the mandible

	mm
P ₃ -M ₃ length	41.0
P ₄ -M ₃ length	35.0
M ₁ -M ₃ length	29.1
Vertical depth of mandibular symphysis	30.0
Antero-posterior thickness of mandibular symphysis	17.0
Depth of mandible at P ₄	29.0
Depth of mandible at M ₂	25.0
Breadth across incisor region	9.0
Thickness of mandibular body at P ₄	13.0
Thickness of mandibular body at M ₂	16.2
Thickness of mandibular body at M ₃	19.6
Inter canine breadth	16.0
Depth of mental foramen below alveolar margin	16.0
P ₄ length	6.5
P ₄ width	6.4
M ₁ length	8.5
M ₁ trigonid width	7.3
M ₁ talonid width	7.6
M ₂ length	10.0
M ₂ trigonid width	9.2
M ₂ talonid width	9.0
M ₃ length	9.6
M ₃ trigonid width	7.8
M ₃ talonid width	7.5

TABLE 2 Miocene and Plio-Pleistocene faunas of Berg Aukas, Namibia

Taxon	Block number								Taxon	Block number							
	BA-47	BA-63	BA-1	BA-31	BA-90	BA-8	BA-54	BA-II		BA-47	BA-63	BA-1	BA-31	BA-90	BA-8	BA-54	BA-II
Primates									Rodentia—continued								
<i>Otaviipithecus</i>			×					×	Nesomyidae								
* <i>Homo sapiens</i>									cf. <i>Notocricetodon</i>	×		×					
Chiroptera									cf. <i>Protarsomys</i>	×	×	×					
indet.					×				cf. <i>Brachyuromys</i>				×				
Megadermatidae									Cricetidae								
<i>Megaderma</i>	×	×							<i>Mystromys</i>						×		×
Hipposideridae									<i>Stenodontomys</i>						×		
<i>Hipposideros</i> small	×	×	×	×					Gerbillidae								
<i>Hipposideros</i> medium	×								<i>Myocricetodon</i>	2	3	2	×	×			
<i>Hipposideros commersoni</i>	?		×	?					<i>Tatera</i>						×	×	
<i>Hipposideros</i> indet.			×	×					<i>Gerbillurus</i>							×	
Rhinolophidae									Cricetomyidae								
<i>Rhinolophus</i>	×	2				2	2		<i>Saccostomus</i>						×		
Vespertilionidae									Dendromuridae								
<i>Myotis</i>		×							cf. <i>Steatomys</i> ?				×				
indet.						×	×		<i>Steatomys</i>						×	×	
Molossidae									<i>Malacothrix</i>						×	×	
indet.		×					×		<i>Dendromus</i>							×	
Insectivora									Muridae								
Erinaceidae									<i>Karnimata</i>				×				
<i>Galerix</i>		×							<i>Mus</i>						×	×	
Soricidae									<i>Aethomys</i>						×		
<i>Crociodura</i>				×	?	2	2		<i>Rhabdomys</i>						2		
Macroscelididae									<i>Michaelamys</i>						×		
indet.	×	×	×	×	×	×	×		<i>Zelotomys</i>						×	×	
Rodentia									<i>Praomys</i>							×	
Thyromyidae									<i>Grammomys</i>							×	
cf. <i>Apodecter</i>	×	×	×	×					<i>Otomys</i>						×	×	
<i>Paraulacodus johanesi</i>				×					<i>Prototomys</i>						×		
Bathyergeridae									Hyracoidea								
<i>Cryptomys</i>						×	×		Procaviidae				×	×			
cf. Anomaluridae		×							Carnivora			×					
Sciuridae									Artiodactyla								
<i>Vulcanisciurus</i>	×	×	×	×					Bovidae								×
indet.	×	×	×	×													
Gliridae																	
<i>Graphiurus</i>						×											

Given that the bulk of the Otavi faunas are rodents, biostratigraphic comparisons are limited to well documented sequences of Neogene rodent faunas. The only 'sequence' in Africa is the series of assemblages from the Maghreb which cover the period from about 12 to 7 Myr⁵⁰. The published East African record is too incomplete for meaningful biostratigraphic comparisons with the Otavi fauna. Thus the most informative comparisons are mainly with European rodent biostratigraphy which is the nearest sequence available for the middle to upper Miocene epoch. Upper Middle Miocene (~lower-middle Astaracian of Europe), block I-47, block I-63; Upper Middle Miocene (~upper Astaracian of Europe), block I-1; lower part of Upper Miocene (~lower Vallesian of Europe), block I-31, block I-90; Upper Pliocene (~Villafranchian of Europe), block I-54; Upper Pliocene–Early Pleistocene (~Villafranchian of Europe), block I-8; Late Pleistocene–Holocene, BA II. cf. Referred to.

* A large fossil hominid femur was purportedly discovered from a level roughly 140 m below surface in a cave near shaft no. 1 (refs 51–53 and A. Palfi, personal communication).

With the exception of Plio-Pleistocene hominids, there are no other hominoids from southern Africa with which to compare *Otaviipithecus*. *Otaviipithecus* is clearly not an australopithecine; moreover, its dental and mandibular morphology and proportions differ from early Miocene small^{4,5} (for example, *Limnopithecus*, *Dendropithecus*, *Micropithecus*, *Kalepithecus*, *Simiolus*) and larger-bodied^{6–10} (for example, *Proconsul*, *Afropithecus*) proconsuloids and oreopithecids (for example *Nyanzapithecus*)¹¹ from East Africa as well as pliopithecids from Eurasia^{12,13}. More appropriate comparisons are with middle Miocene hominoids of East Africa and Eurasia such as *Kenyapithecus*, *Sivapithecus* and *Dryopithecus*.

Kenyapithecus and the closely related *Sivapithecus* are known from several sites in Kenya and Eurasia^{1,14–28}. Although it is unwise to overgeneralize about these two genera because of their dento-gnathic variability, they share with *Otaviipithecus* such features as: (1) reduced molar cingula; (2) narrow symphyseal regions; (3) well-developed mandibular postcanine fossae; and (4) accessory cusplets on M₃s. But both *Kenyapithecus* and

Sivapithecus differ from *Otaviipithecus* in having one or more of the following features: (1) thickened and rounded inferior transverse tori and reduced superior transverse tori (particularly *Kenyapithecus*); (2) poorly developed submandibular fossae; (3) canine roots that converge toward the mandibular symphysis (particularly *Kenyapithecus*); (4) robust anterior roots of P₃s; (5) buccal flare on P₄s (particularly *Sivapithecus*); (6) marked differential wear on molars; (7) thickened molar enamel and low dento-enamel relief; (8) more robust mandibular bodies; (9) ascending mandibular rami that sweep upward from below M₂ and hide most of M₃ in lateral view; and (10) M₃s that are larger than M₂s.

Dryopithecus is best known from sites in southern Europe^{29–33}. *Otaviipithecus* and *Dryopithecus* share several characteristics such as: (1) relatively thin enamel; (2) reduced or absent buccal cingulum; and (3) moderately developed inferior transverse tori. But there are some important distinctions. For example, *Dryopithecus* differs from *Otaviipithecus* in having: (1) relatively deep and narrow mandibular corpora that shallow posteriorly to a

marked degree; (2) relatively large anterior and posterior foveas on the molars; (3) distally placed hypoconulids; (5) buccolingually constricted molar cusps; (6) more sectorial P_3 s; (7) more marginally placed molar cusps with larger, open basins; and (8) less steeply inclined alveolar planes^{25,29,31,32}.

Regression equations relating molar size to body weight in modern anthropoids suggest that *Otaviopithecus* probably weighed between 14–20 kg, that is about the same body size estimated for *Proconsul africanus* or *Dryopithecus laietanus*³⁴ but smaller than the 30–35 kg of female *Pan paniscus*³⁵. The minimal differential wear on the molars, combined with the thinness of the enamel, suggests that *Otaviopithecus* probably subsisted on nonabrasive foods. The narrowness of the incisor region also argues against a specialized frugivorous diet of tough-skinned fruits³⁶, but neither molar size nor shape show any adaptations to a specialized folivorous diet^{37,38}. It is most likely that *Otaviopithecus* subsisted on foods such as leaves, berries, seeds, buds and flowers (foods not needing much incisal preparation before mastication). Because the timing of dental emergence, as inferred from molar wear patterns, is related to the length of the maturational process in major primate grades, the molars of *Otaviopithecus* probably emerged in rapid succession as they do in living chimpanzees^{39–41}. Estimation of the age at death using chimpanzee maturation rates suggest that this individual died at about 10 years of age.

We cannot be certain about the phylogenetic position of *Otaviopithecus*, but the most likely possibilities (in decreasing order of likelihood) are that *Otaviopithecus* represents the sister group of: (1) Miocene large-bodied hominoids; (2) the African ape-hominid clade; (3) the African ape clade; or (4) the *Australopithecus-Homo* clade.

The breccias at Berg Aukas and its vicinity yield rich samples of vertebrates, particularly micromammals (Table 2)⁴². The relatively high proportion of micromammals in the Miocene breccias suggests that the fossil accumulations were largely due to the activities of raptors (both diurnal birds of prey and owls) and/or as the result of accidental falls in caverns or sink holes by animals living in the vicinity of the cave mouths. Of 37 mammal species thus far recorded in the Miocene breccias of Otavi, 20 are nocturnal, 4 are diurnal, 1 is both, and 12 are of unknown

daily rhythms. The preponderance of nocturnal species probably reflects the origin of the samples as regurgitation pellets of owls, or of bats which died in the caves. Medium and large mammal remains are rare, but among those found was a partial skeleton of a hyracoid. Preliminary faunal studies of the Miocene breccias suggest that the Otavi region was more humid during the middle and upper Miocene than it is today. This contrasts with the faunas found in the Plio-Pleistocene breccias which indicate a semi-arid environment in which gerbils and other rodents adapted to xeric conditions were more common (Table 2). At present, the 'karstveld' (2,000 m altitude) is covered with highland savanna characterized by the trees *Syringa*, *Moringa*, fig trees, *Marula*, leadwood, sickle, *Terminalia* and the succulent shrub, *Aloe*, with patches of high annual and perennial grasses⁴³, a flora most unsuitable for Miocene hominoids.

The only other Miocene mammals known from southern Africa also come from Namibia, in coastal deposits of the Sperrgebiet ('Forbidden Territory') south of Lüderitz^{44–47}, and from the Orange River terraces at Arrisdrift^{48,49}. Namibia is thus an important region for information about vertebrate evolution, palaeoclimates, and ecological changes in southern Africa over the past 15–16 million years. □

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- Alpagut, B., Andrews, P. & Martin, L. *J. hum. Evol.* **19**, 397–422 (1990).
- Conroy, G. C. *Palaeont. afr.* **28**, 53–58 (1991).
- Keene, H. J. *Am. J. phys. Anthropol.* **25**, 208–209 (1966).
- Harrison, T. *Folia Primatol.* **50**, 59–108 (1988).
- Leakey, R. E. & Leakey, M. G. *J. hum. Evol.* **16**, 369–387 (1987).
- Andrews, P. *Bull. Br. Mus. nat. Hist.* **30**, 85–224 (1978).
- Andrews, P. in *Ancestors: The Hard Evidence* (ed. Delson, E.) 14–22 (1985).
- Andrews, P. J. & Martin, L. B. *J. hum. Evol.* **16**, 101–118 (1987).
- Leakey, R. E., Leakey, M. G. & Walker, A. C. *Am. J. phys. Anthropol.* **76**, 277–288 (1988).
- Leakey, R. E., Leakey, M. G. & Walker, A. C. *Am. J. phys. Anthropol.* **76**, 289–307 (1988).
- Harrison, T. *Am. J. phys. Anthropol.* **71**, 265–284 (1986).
- Harrison, T., Delson, E. & Jian, G. *J. hum. Evol.* **21**, 329–362 (1991).
- Ginsburg, L. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 47–57 (Cambridge University Press, Cambridge, 1986).
- Matsuda, T. et al. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 35–46 (Cambridge University Press, Cambridge, 1986).
- Pickford, M. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 19–34 (Cambridge University Press, Cambridge, 1986).
- Ishida, H., Pickford, M., Nakaya, H. & Nakano, Y. *Aft. Studies. Monog. suppl.* **2**, 73–85 (1984).
- Pickford, M. *Am. J. phys. Anthropol.* **58**, 1–19 (1982).
- Pickford, M. *J. hum. Evol.* **14**, 113–143 (1985).
- Pickford, M. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 163–172 (Cambridge University Press, Cambridge, 1986).
- Bernor, R. L. & Tobien, H. *J. hum. Evol.* **19**, 551–568 (1990).
- Barry, J. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 93–106 (Cambridge University Press, Cambridge, 1986).
- Pilbeam, D. R. et al. *Nature* **270**, 689–695 (1977).
- Raza, S. et al. *Nature* **306**, 52–54 (1983).
- Kay, R. F. & Simons, E. L. in *New Interpretations of Ape and Human Ancestry* (eds Ciochon, R. L. & Corruccini, R. S.) 577–624 (Plenum, New York, 1983).
- Kelley, J. & Pilbeam, D. in *Comparative Primate Biology* (eds Swindler, D. R. & Erwin, J.) 361–411 (Liss, New York, 1986).
- Pilbeam, D., Rose, M., Badgley, C. & Lipschutz, B. *Yale Peabody Mus. nat. Hist.* **181**, 1–94 (1980).
- Kappelman, J. et al. *J. hum. Evol.* **21**, 61–73 (1991).
- Benefit, B. R. & McCrossin, M. L. *J. hum. Evol.* **18**, 493–497 (1989).
- Begun, D. R., Moya-Sola, S., Kohler, M. *J. hum. Evol.* **19**, 255–268 (1990).
- Mein, P. in *Primate Evolution* (eds Else, J. G. & Lee, P. C.) 59–70 (Cambridge University Press, Cambridge, 1986).
- Begun, D. R. *J. hum. Evol.* **20**, 521–526 (1991).
- Harrison, T. *J. hum. Evol.* **20**, 515–520 (1991).
- Simons, E. L. & Pilbeam, D. R. *Folia Primatologica* **3**, 81–152 (1965).
- Conroy, G. C. *Int. J. Primatol.* **8**, 115–137 (1987).
- Jungers, W. L. in *Size and Scaling in Primate Biology* (ed. Jungers, W. L.) 345–381 (Plenum, New York, 1985).
- Hylander, W. L. *Science* **189**, 1095–1098 (1975).
- Lucas, P. W., Corlett, R. T. & Luke, D. A. Z. *Morph. Anthropol.* **76**, 253–276 (1986).
- Kay, R. F. in *Adaptations for Foraging in Nonhuman Primates* (eds Rodman, P. S. & Cant, J. G. H.) 21–53 (Columbia University Press, New York, 1984).
- Conroy, G. C. & Mahoney, C. J. *Am. J. phys. Anthropol.* **86**, 243–254 (1991).
- Conroy, G. C. & Vannier, M. W. *Am. J. phys. Anthropol.* **86**, 137–156 (1991).
- Conroy, G. C. & Vannier, M. W. *Am. J. phys. Anthropol.* **86**, 121–136 (1991).
- Senut, B., Pickford, M., Mein, P., Conroy, G. & Van Couvering, J. *C. r. heb. Acad. Sci., Paris* (in the press).
- Schlechter, K. *Primary Atlas for Namibia*. (Maskew Miller, Windhoek, 1987).
- Hopwood, A. T. *Am. Mus. Novit.* **344**, 1–9 (1929).
- Stromer, E. in *Die Diamantenwüste Südwest-Afrikas* (ed. Kaiser, E.) 107–153. (Reimer, Berlin, 1926).
- Pickford, M. *Tertiary Res. Spec. Pap.* **7**, 1–83 (1986).
- Van Couvering, J. A. & Hamilton, W. R. *Nat. geogr. Soc. Res. Reports*, 1975 697–703 (1983).
- Corvinus, G. & Hendey, Q. B. *N. Jb. Geol. Palaeont. Mh.* **4**, 193–205 (1978).
- Pickford, M. *Ann. S. Afr. Mus.* **97**, 283–295 (1987).
- Coiffait-Martin, B. *Contribution des Rongeurs du Néogène d'Algérie à la Biochronologie Mammalienne d'Afrique Nord-Occidentale* (University Nancy, France, 1991).



FIG. 2 Distribution of middle and late Miocene Hominoidea (black dots) and extant African apes (stippling). Asterisk indicates site of *Otaviopithecus* discovery.

51. Mason, R. *Ann. S. Afr. Mus.* **71**, 215–223 (1976).
 52. Malan, M. 'Thigh bone discovery excites the experts' *The Star* (Johannesburg, 7 February 1975).
 53. Sandelowsky, B. *Am. Sci.* **71**, 606–615 (1983).

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Novel major archaeobacterial group from marine plankton

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MARINE bacteria often dominate the plankton biomass^{1,2} and are responsible for much of the cycling of organic matter³, but bacterial diversity is poorly understood because conventional identification methods (requiring culturing) miss about 99% of the organisms^{4,5}. Recent advances permit characterization of microbial communities by analysis of 16S ribosomal RNA gene sequences directly from biomass without the need to culture the organisms⁶; such studies from surface ocean samples have found only eubacteria^{7–10}, not archaeobacteria (or Archaea¹¹), which are profoundly different¹². Here we report 16S rRNA sequences obtained from Pacific Ocean bacterioplankton samples collected from depths of 100 m and 500 m. Among these we found sequences only distantly related to those of any organisms previously characterized by 16S rRNA sequences, with similarities to the nearest such relatives (extreme thermophiles) approximately the same as those between animals and plants. We suggest that these sequences are from a previously undescribed archaeobacterial group that may have diverged from the ancestors of characterized organisms very early in evolution.

Our samples were collected from the western side of the California Current, roughly 350 miles west of San Diego. The area was oligotrophic at the time of sampling, as indicated by the low surface chlorophyll concentrations and a chlorophyll maximum layer at about 100 m depth, near the base of the euphotic zone and substantially below the surface mixed layer (Fig. 1). The samples for genetic analysis came from this biologically active layer (100 m depth) as well as from 500 m; the deeper sample was substantially below the euphotic zone and possessed a significantly lower total bacterial abundance and temperature (Fig. 1). Comparison of 16S rRNA gene sequences from these samples to those from similarly characterized organisms reveals that five of the clones from the 500-m sample and two from a 100-m sample form a cluster within the archaeobacteria, yet this cluster is only distantly related (about 70% sequence identity with 16S rRNA) to any previously described archaeobacterial group (Fig. 2). The sequences most similar to these clones come from extreme thermophiles, such as *Pyrodicticum*. Measurable hybridization (under stringent conditions) of a probe made from one of the clones (NH49-8) to nucleic acid from the archaeobacterium *Sulfolobus acidocaldarius*, but not that from the eubacterium *Escherichia coli* or eukaryotic calf thymus, was also consistent with the inferred archaeobacterial origin of these clones.

A majority (5 out of 7) of our clones from 500 m were members of this group, as were a small minority (2 clones out of 10) from only 1 of 3 different 100-m samples collected a few days apart (>30 clones from 100 m were examined; information on other clones will be reported elsewhere). Because the distribution of clones in final polymerase chain reaction (PCR) products may

not match the distribution of all 16S rRNA genes in the original unamplified DNA (owing to differences in amplification efficiency), we cannot yet say how prevalent these organisms are in sea water. This may best be determined by hybridization of specific oligonucleotide probes to RNA isolated from the natural communities⁷ (not possible with these samples owing to the limited material remaining). But community DNA from the 500-m sample hybridized very strongly to DNA from 1,000 m (ref. 13), suggesting that the dominant organisms from 500 m may dominate a large depth range. It is therefore possible that this new group may be extremely abundant, and may be a significant component of deep-sea metabolism.

At this point in our studies, we know nothing about these organisms other than their 16S rRNA sequences. Although the closest characterized apparent relatives are extreme thermophiles, the temperature of the water from which our samples came was 5–15 °C, and remote from any known hydrothermal sources. Also, the G + C content, which correlates with thermal stability, of our longest clones (NH49-8 and -9) was only ~51%, compared with 63% for *Sulfolobus* over the same region. Therefore, we think the sequences probably do not come from thermophiles. Phylogenetic statistical evaluation of the sequence data suggests we cannot at this time confidently place these clone sequences within the extreme thermophile branch of the archaeobacteria. A bootstrap analysis, which involves creation of many phylogenetic trees from random resampling within the sequence¹⁴, and with the eubacterium *Deinococcus radiodurans* as the outgroup, indicated that although the closest relative to our clones appears to be *Pyrodicticum occultum*, the branch that leads to our clones does not consistently diverge with the extreme thermophiles; in 40% of 70 trees generated by the bootstrap analysis, the branch leading to our clones diverged within the

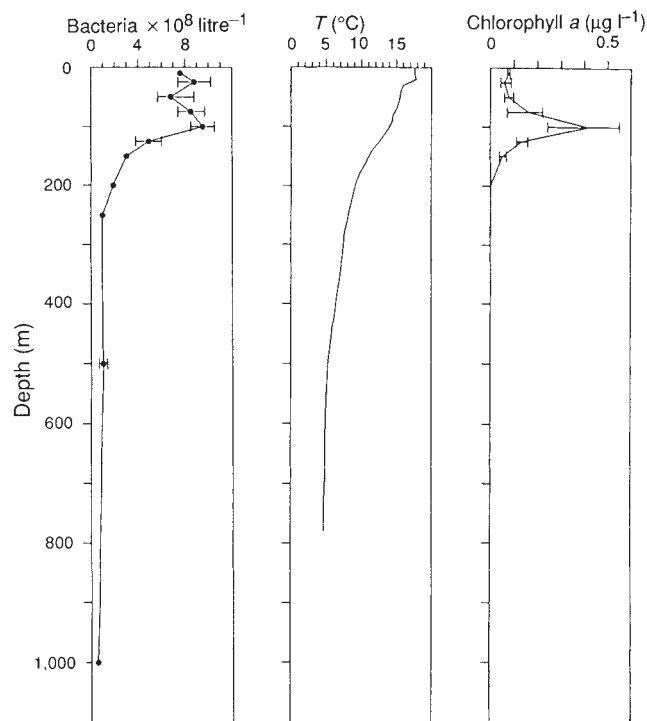
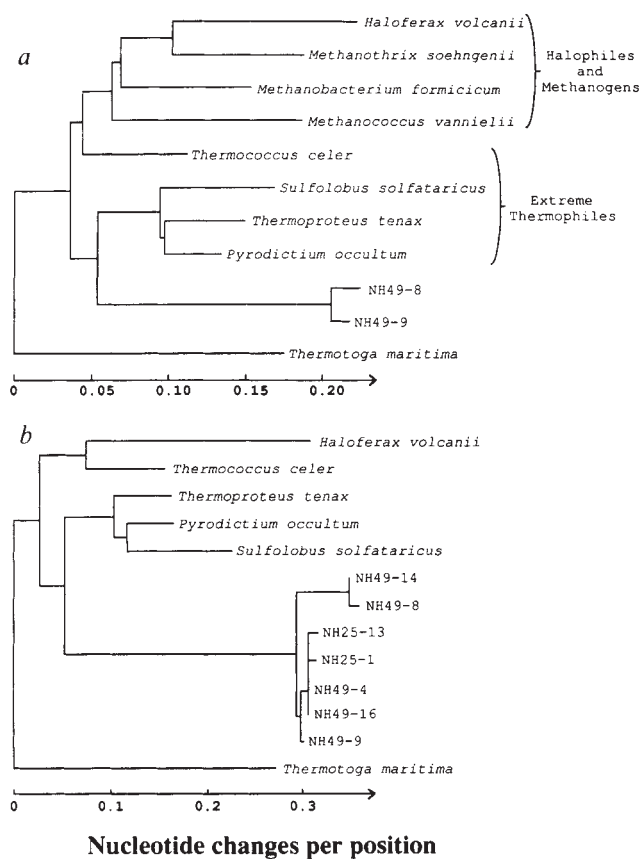


FIG. 1 Depth profiles of parameters at sampling location (within 2 km of 31° 50' N 124° 6' W). Error bars indicate range of values of the 7-day sampling period (21–27 April 1989) for the biological parameters; points without error bars were not replicated. Temperature profile was measured on 22 April 1989. Bacterial abundance was measured by epifluorescence microscopy with acridine orange stain¹⁸, particulate chlorophyll *a* collected on 0.45-µm-pore-size filters was analysed by fluorometry¹⁹ and temperature measured by an expendable bathythermograph.

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extreme thermophile lineage, in 36% the divergence occurred within the methanogen/halophile lineage, and in 24% the branch diverged closer to the eubacteria than the extreme thermophile split from the methanogen/halophile lineage. Therefore we cannot rule out that these organisms may be methanogens (common in anaerobic environments, so perhaps released from anaerobic guts of metazoa; the water is not anaerobic in this region), but the sequences are clearly distinct from all known groups of methanogens^{11,12} (Fig. 2). Learning more about the organisms will probably require enrichment or isolation of such organisms. The sequences described here include several regions suitable for use as specific probes; therefore, many enrichment conditions can be tried and these can be screened with such probes to see which conditions, if any, enhance growth of this group. Given that other archaeobacteria usually thrive under conditions in

FIG. 2 Phylogenetic associations, based on 16S rRNA sequences, of clones and previously characterized organisms. All organisms shown are archaeobacteria except for *Thermotoga maritima* (a eubacterium¹²). Clones beginning NH49- came from particulate DNA collected from 500 m depth on 27 April 1989, and those beginning NH25- from such DNA collected from 100 m depth on 23 April 1989; numbers after hyphens are arbitrary clone numbers. *a*, Representatives of known archaeobacterial groups and clones NH49-8 and NH49-9, comparing 781 bases, essentially the full-length clone excluding regions of ambiguous alignment. *b*, Analysis of several marine clones, comparing 200 bases (both regions beginning at *E. coli* base number 537). Samples (100–200 litres) were collected in 30-litre Niskin bottles on a rosette sampler (General Oceanics, Miami). Microorganisms were filtered onto a 142-mm diameter 0.22- μ m-pore-size Durapore filter (Millipore) after prefiltration through a Gelman A/E glass fibre filter that removed almost all eukaryotes and about 5–10% of the bacteria²⁰. DNA was extracted with hot 1% sodium dodecyl sulphate and purified by precipitation with ethanol and extraction with phenol²¹. Partial genes encoding 16S rRNA were amplified from 1 ng DNA by PCR²² with a GeneAmp Kit and recombinant *Taq* polymerase (Perkin Elmer-Cetus) and 1 μ M each of the primers TTGAGCTCAA-GCTTCAGCA/CGCCGCGGTAATA/TC AND TTTTGGATCCTCTAGAACGGCGGT-GTGTA/GC (these consist of linkers at the 5' ends and 'universal' 16S rRNA sequences²³, located at *E. coli* position numbers 537 and 1,390, at the 3' ends), over 30 cycles with the following temperature program: 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min. Negative controls (water instead of DNA) showed no amplification. Products of the proper size range (~950 base pairs) were cut out of a low-melting-agarose gel (SeaKem GTG), purified by extraction with phenol and precipitation with ethanol, digested with *Bam*H1 and *Hind*III, and cloned into M13 (ref. 24), then sequenced by the dideoxy chain termination method with Sequenase 2 (US Biochem.). Sequences were analysed by the distance matrix method²⁵. Sequences from known organisms came from GenBank²⁶. The analysis in *a* used the following base positions, numbered from the 5' end of the aligned *Thermoproteus tenax* sequence: 496–498, 500–799, 803–806, 810–897, 899–906, 914–954, 1,002–1,071, 1,073–1,088, 1,090–1,091, 1,096–1,099, 1,104–1,245, 1,247–1,248, 1,250–1,354. Secondary structure analysis suggested that potential PCR artefacts did not affect the results significantly, as differences between clones usually included compensatory changes on opposite sides of base-paired regions^{7,8}; also, a phylogenetic tree essentially the same as *a* was obtained with the first 400 bases or the last 400 bases of clones NH49-8 and NH49-9. Sequences from the clones reported here have been submitted to EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers Z11568–Z11573.

which there is little competition from eubacteria or eukaryotes (for example, high temperatures, low pH, high salt), it would be interesting to learn what niche these organisms fill in the sea. They may also be of interest phylogenetically because they appear to have diverged early in the evolution of life and have their closest characterized relatives within a broad group that until now was thought to consist only of extreme thermophiles¹¹.

Although it is unsatisfactory to have only a gene sequence, we now know there is something interesting to look for, and we have a probe to assist in the search. The approach we used to discover new microbial groups supplements traditional microbiological techniques^{6–10,15–17}, with this novel archaeobacterial group appearing in the first few midwater plankton samples investigated. □

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1. Cho, B. C. & Azam, F. *Nature* **332**, 441–443 (1988).
2. Fuhrman, J. A., Sliester, T. D., Carlson, C. A. & Proctor, L. M. *Mar. Ecol. Prog. Ser.* **57**, 207–217 (1989).
3. Azam, F. *et al. Mar. Ecol. Prog. Ser.* **10**, 257–263 (1983).
4. Jannasch, H. W. & Jones, G. E. *Limnol. Oceanogr.* **4**, 128–139 (1959).
5. Ferguson, R. L., Buckley, E. N. & Palumbo, A. V. *Appl. envir. Microbiol.* **47**, 49–55 (1984).
6. Pace, N. R., Stahl, D. A., Lane, D. L. & Olsen, G. J. *Adv. microb. Ecol.* **9**, 1–55 (1986).
7. Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. & Field, K. G. *Nature* **345**, 60–63 (1990).
8. Giovannoni, S. J., DeLong, E. F., Schmidt, T. M. & Pace, N. R. *Appl. envir. Microbiol.* **56**, 2572–2575 (1990).
9. Britschgi, T. B. & Giovannoni, S. J. *Appl. envir. Microbiol.* **57**, 1707–1713 (1991).
10. Schmidt, T. M., DeLong, E. F. & Pace, N. R. *J. Bact.* **173**, 4371–4378 (1991).
11. Woese, C. R., Kandler, O. & Wheelis, M. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4576–4579 (1990).
12. Woese, C. R. *Microb. Rev.* **51**, 221–271 (1987).
13. Lee, S. & Fuhrman, J. A. *Limnol. Oceanogr.* **36**, 1277–1287 (1991).
14. Felsenstein, J. *Evolution* **39**, 783–791 (1985).
15. Distel, D. L. *et al. J. Bact.* **170**, 2506 (1988).
16. Ward, D. M., Weller, R. & Bateson, M. M. *Nature* **345**, 63–65 (1990).

17. Amann, R. *et al. Nature* **351**, 161–163 (1991).
18. Hobbie, J. E., Daley, R. J. & Jasper, S. *Appl. envir. Microbiol.* **33**, 1225–1228 (1977).
19. Holm-Hansen, O. *et al. J. Cons. perm. int. Explor. Mer.* **30**, 3 (1985).
20. Lee, S. & Fuhrman, J. A. *Appl. envir. Microbiol.* **56**, 739–746 (1990).
21. Fuhrman, J. A., Comeau, D. E., Hagstrom, J. & Chan, A. M. *Appl. envir. Microbiol.* **54**, 1426–1429 (1988).
22. Saiki, R. K. *et al. Science* **230**, 1350–1354 (1985).
23. Lane, D. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 6955–6959 (1985).
24. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York, 1982).
25. Olsen, G. J. *Meth. Enzym.* **164**, 793–812 (1988).
26. Blifsky, H. S. & Burks, C. *Nucleic Acids Res.* **16**, 1861 (1988).

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Receptive field dynamics in adult primary visual cortex

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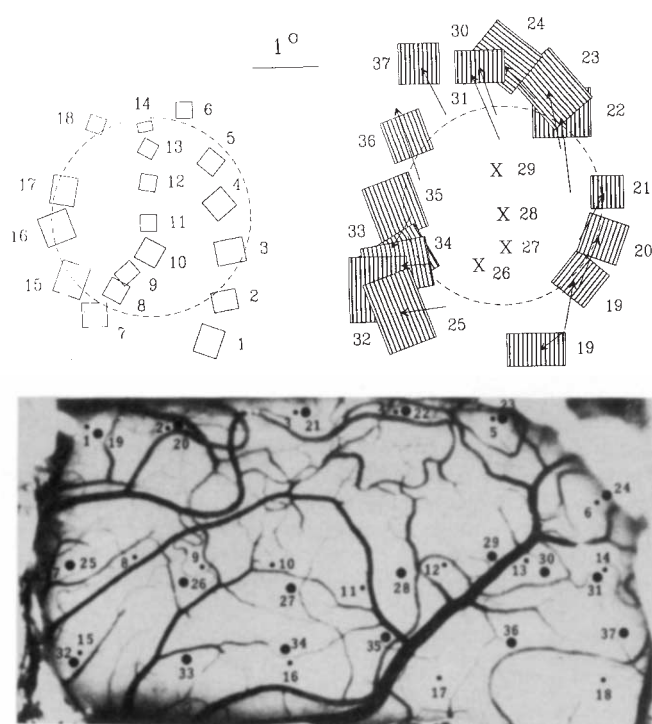
THE adult brain has a remarkable ability to adjust to changes in sensory input. Removal of afferent input to the somatosensory, auditory, motor or visual cortex results in a marked change of cortical topography¹⁻¹⁰. Changes in sensory activity can, over a period of months, alter receptive field size and cortical topography¹¹. Here we remove visual input by focal binocular retinal lesions and record from the same cortical sites before and within minutes after making the lesion and find immediate striking increases in receptive field size for cortical cells with receptive fields near the edge of the retinal scotoma. After a few months even the cortical areas that were initially silenced by the lesion recover visual activity, representing retinotopic loci surrounding the lesion. At the level of the lateral geniculate nucleus, which provides the visual input to the striate cortex, a large silent region remains. Furthermore, anatomical studies show that the spread of geniculocortical afferents is insufficient to account for the cortical recovery. The results indicate that the topographic reorganization within the cortex was largely due to synaptic changes intrinsic to the cortex, perhaps through the plexus of long-range horizontal connections.

By comparing receptive field size and position for identical cortical sites in the primary visual cortex monitored before,

immediately after (Fig. 1) and months after (Figs 2 and 3) making the retinal lesions, we were able to determine the nature of the changes occurring in the short and long term. Dramatic changes were observed even minutes after the lesion: those sites with receptive fields located close to the centre of the lesion were made unresponsive, but at recording sites where the fields were originally close to and just inside the lesion boundary, there was a large increase in receptive field area. In the experiment illustrated in Fig. 1, receptive field maps of cells in the superficial cortical layers recorded before making the lesion expanded fivefold in area. The expanded fields were well outside the normal range of field sizes of superficial layer cells at this eccentricity. Though the change in field size was the most obvious effect, there was also a small centrifugal shift in field positions from the centre of the lesion. The shift might simply reflect field enlargement combined with a removal of one side of the field by the lesion, resulting in an apparent shift. Though slight shifts of field position do tend to occur as a result of eye movement, in this and other experiments we revisited a subset of the sites (about 1/5 of the total) before lesioning to establish the repeatability of the measurements of size and position, and demonstrated their stability within the recording session relative to the changes observed following the lesion. Furthermore, the consistent centrifugal shifts in position argue against attributing them to random eye movements. Results analogous to our observations in visual cortex were reported in the flying fox somatosensory cortex following digit amputation¹², and in motor cortex following sensor-motor nerve transection^{4,13}, with immediate changes in cortical topography and, in somatosensory cortex, increase in receptive field size as well. In our experiments the properties of the expanded receptive fields were similar to

FIG. 1 Receptive fields of cells encountered in vertical electrode penetrations in the superficial layers of monkey area V1 before and immediately after binocular retinal lesions at retinotopically corresponding sites. The first sets of receptive field maps, made before the lesion, are shown on the top left, with the subsequently made lesion included for reference (dashed lines). The size and retinotopic positions of the receptive fields encountered in one animal within minutes after making the lesion are shown at the top right. Using the cortical vasculature for reference (bottom), the same recording sites made before the lesion (small dots) and after the lesion (large dots) are numbered, with the corresponding receptive fields numbered accordingly. On the same day of the lesion a number of the originally recorded cortical sites were unresponsive to visual stimuli (as indicated by the Xs on the right figure). The most striking effect was that receptive fields originally located near the boundary of the lesion have greatly expanded, on average reaching 5 times their original area: before the lesion, the average field area at the recording sites was 0.07 deg^2 ($\pm 0.03 \text{ deg}^2$), and 0.37 deg^2 ($\pm 0.26 \text{ deg}^2$) immediately after the lesion was made ($p < 0.01$). In addition, there was a suggestion of a shift in receptive field position from immediately inside, to just outside the boundary of the lesion, although this shift was less than 1° . The arrows in the top right indicate the relative positions of the receptive field centres of cells at nearby cortical sites recorded before and after the lesion, with the starting centre position of each receptive field indicated by the tail end of the arrow, and the ending position by the arrow head. A few of the arrow positions were interpolated at points where the before and after recording sites were not sufficiently close. The position of the fovea is indicated by the Xs at the top of the figure.

METHODS. Electrophysiology and lesioning procedures were done in anaesthetized, paralysed cats and monkeys (anaesthetic, sodium pentothal; paralytics, Norcuron for monkeys, succinylcholine for cats; anaesthesia level monitored by electroencephalography). A total of 12 pairs of lesions were made at corresponding sites in the two retinæ of 6 monkeys and 8 pairs of lesions in 4 cats. Receptive field maps, made with hand-held stimulator, are 'minimum response' fields for cells in the superficial layers, obtained with a light bar generated by a hand-held projector, and were mapped independently for each eye. In each experiment, a selected region of cortex was mapped, within this region a site was chosen so that the receptive fields would be centred within the lesion, which was made in the parafoveal retina with a diode laser directed through an indirect ophthalmoscope (Iris Instruments; $400 \text{ mW} \times 500 \mu\text{s}$). Lesions of this strength and duration



destroyed primarily the outer retina, leaving the retinal ganglion cell layer intact, as later determined by post mortem histology in each animal. In monkeys, the lesions were centred 3 to 5° below the fovea near the midline, and were 1 mm , or 3 to 5° in diameter. In cortical terms, this produced a region of unresponsive cells $\sim 8 \text{ mm}$ in diameter. The visuotopic boundaries of the lesions were mapped by back projecting the beam from a Nikon fundus camera to the tangent screen on which the receptive fields were mapped, enabling us to determine the positions of the receptive fields relative to the lesion. Using the cortical vasculature for reference, another series of electrode penetrations was made at the original mapping sites immediately following the lesion.

those seen in normal cortex, including orientation selectivity, directionality and binocularity.

To compare the long term effects of laser lesions with those observed on the same day as the lesion, we followed the course of receptive field structure and cortical topography for two months following the lesion. At the end of this period, all cortical sites could be activated by visual stimuli, but in comparison with the map made before the lesion, we observed large shifts in field position, up to 5° , for those recording sites with fields originally located at the centre of the lesion (Fig. 2). As described previously, this resulted in a great expansion of the representation of the perilesion retina⁸⁻¹⁰. In several experiments we observed shifts in field position for cells situated at cortical sites originally representing parts of the retina outside the lesion. Other receptive field anomalies were also observed, including bipartite receptive fields spanning the lesion, fields in the ipsilateral visual field (presumably generated by callosal input), and shifts in different directions for the two eyes. These effects and the accumulation of fields at the edge of the lesion would not be seen in the cortices of unlesioned animals. For the experiment illustrated in Fig. 2, the receptive fields in the recovered cortex were severalfold larger than normal. The ratio of expansion was not as great as that seen immediately after the lesion, suggesting that the initial expansion is followed by a consolidation in the long term.

The alterations of cortical topography in adult animals first seen in the somatosensory cortex following digit amputation have now been demonstrated in studies of various sensory and motor areas¹⁻¹⁰. A persistent question is whether the locus of change is at the cortical level or at a prior stage in the sensory pathway. Earlier studies have shown topographical shifts at the subcortical level resulting from deafferentation¹⁴. By the same token, retinal lesions can change the topography of the lateral geniculate nucleus (LGN), though the changes shown previously were small (limited to $200\ \mu\text{m}$)¹⁵. By comparing the topographical changes in the LGN and cortex in the same animal, at the same time and with the same type of lesion, we were able to determine that most of the long-term cortical changes seen in the current study originated in the cortex: in two cats (the results from one are illustrated in Figs 3 and 4) and one monkey, at a time when the initial cortical scotoma had disappeared (Fig. 3), there was still a large unresponsive area in the LGN (about 1 mm in diameter, corresponding topographically to the size of the retinal scotoma). The expected extent of the affected area in the LGN was determined by making two injections of different retrograde tracers in the cortex on either side of the original cortical scotoma. Electrode penetrations between the clusters of labelled cells in the LGN showed no visually activated cells (Fig. 4c). None of the other features of the cortical reorganization, such as an enlarged representation of perilesion retina, enlarged or bipartite fields, was observed in the LGN (Fig. 4a). Finally, the lack of overlap in the distributions of the two clusters

FIG. 3 Mapping of cat visual cortex 2 months after binocular retinal lesions. At this time the cortical scotoma has entirely filled in. The right of the figure shows the receptive field maps and lesion boundary, and the left part shows the Horsley-Clarke coordinates of the recording sites (mm), which are numbered. The initial cortical scotoma was centred at site 7. With regular shifts in recording position from posterior to anterior (sites 1 to 7, 18 and 19) and from lateral to medial (sites 17 to 7, lying in area 18, and sites 7 to 15 corresponding to a tangential electrode penetration down the medial bank of the lateral gyrus, within area 17) all sites were activated by visual stimuli. Nearby sites (5 and 6) had fields lying on opposite sides of the lesion, and the receptive field positions tended to accumulate around the perilesion retina. Other field anomalies were observed, including bipartite fields, with subfields lying on either side of the scotoma (8 and 9), fields lying in the ipsilateral visual field (3), and fields in different positions in the two eyes (not shown). The receptive field maps and lesion boundary

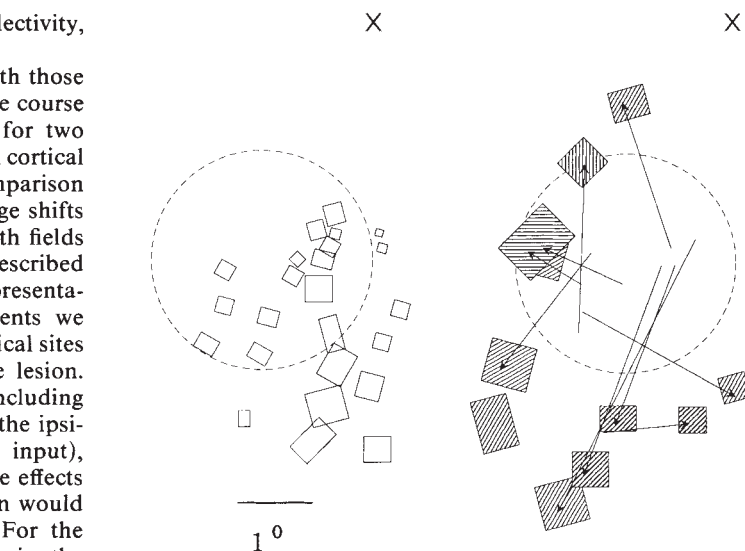
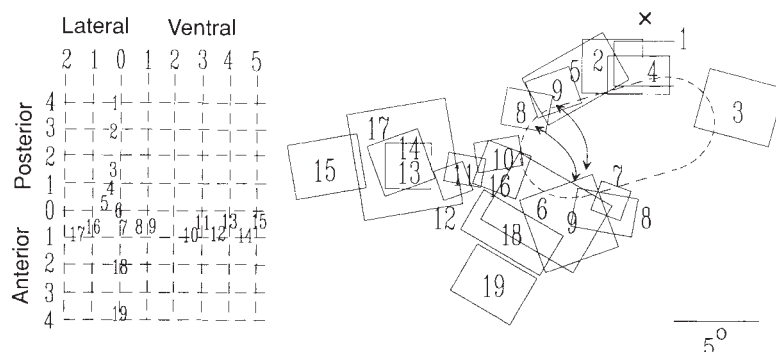


FIG. 2 Receptive field maps in a region of monkey cortex deafferented by a retinal lesion, immediately before the lesion was made (left) and two months following the lesion (middle). This was done in a different animal from the one illustrated in Fig. 1. A much larger shift in receptive field position is seen at two months than in the immediate-term experiments, with the cortical scotoma having entirely filled in. All recorded sites had cells with receptive fields located outside the lesioned retinal area, although a few fields did overlap with the scotoma, probably owing to incomplete destruction at the edge of the scotoma in the retina. Although some arrows are crossed, overall the shifts maintained a rough retinotopic order, with fields that were originally located in the lower part of the scotoma shifting down, and those located in the upper part shifting up. Note that for one site where the receptive field was initially located outside the lesion, the field shifted horizontally. This result was observed in several experiments, and indicates that the effects of the perturbation caused by the lesion are propagated beyond the deafferented area of cortex. There was also receptive field enlargement, though less than that observed in the short-term experiment shown in Fig. 1: the field areas averaged $0.036\ \text{deg}^2$ ($\pm 0.022\ \text{deg}^2$) before the lesion and $0.100\ \text{deg}^2$ ($\pm 0.025\ \text{deg}^2$) two months later ($P \ll 0.01$). The Xs mark the foveal position.

of labelled cells suggested that the fill in of the cortical scotoma was not mediated either by the normal spread of geniculate afferents within the cortex, or by their sprouting into the cortical scotoma.

The transmission of visual information from one visual field locus to another, taking place at both the immediate and long term in the cortex, may instead be mediated by the long-range horizontal connections¹⁶⁻²⁰. These connections have been implicated in dynamic, contextually dependent changes in the



(dashed line) are shown for one eye only. X marks the centre of the area centralis.

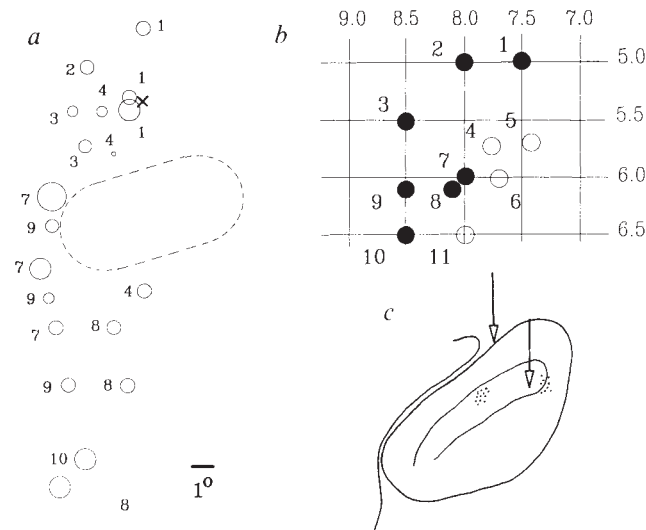


FIG. 4 Mapping of cat lateral geniculate nucleus in the same animal showing fill-in of the cortical scotoma illustrated in Fig. 3. At the two-month time point, a 1-mm wide silent area remained in the LGN. *a*, Receptive field maps of cells encountered in multiple penetrations shown in *b*. In penetration 4, there was a regular shift in receptive field position up to the edge of the scotoma, followed by a 1-mm-long zone of silence, followed by a resumption of activity on the other side of the scotoma. None of the characteristics seen in the recovered cortex, such as an overrepresentation of the perilesion retina, enlarged fields at the edge of the scotoma or bipartite fields, was seen in the LGN. *b*, Positions of penetrations in the LGN in Horsley-Clarke coordinates (mm). Several of the penetrations, covering an area ~1 mm wide, encountered visually unresponsive cells within the geniculate (open circles). Surrounding this area the electrodes encountered visually responsive cells (closed circles). *c*, Coronal view of a section through the LGN. Injections of red and green fluorescent latex microspheres (Lumafluor) were made on either side of the original cortical scotoma after the two-month survival to label the expected boundaries of the scotoma in the LGN (clusters of dots). The mapping and histology were done two weeks after the injections. Penetrations between the clusters at this antero-posterior level (open arrows) encountered visually unresponsive cells, demonstrating a sizable retained geniculate scotoma at a time when all parts of the cortex could be activated by visual stimuli. The lack of overlap between the clusters suggests further that the filling in seen in cortex is not likely to be mediated by the spread of geniculate afferents within the cortex.

response properties of cortical cells²¹. Our results suggest that the subthreshold influences of horizontal connections can be potentiated to be capable of activating the cell. The magnitude of the topographical shifts, roughly 4 to 5 mm, are large relative to the lateral spread of thalamic afferents, but the horizontal connections have an extent sufficient to account for such a reorganization. Furthermore, they are specific in connection columns of similar orientation specificity²²⁻²⁴, and could explain the orientation selectivity observed in the reorganized cortex. The receptive fields seen in the ipsilateral visual field are likely to come from the contralateral hemisphere by way of the corpus callosum.

Perhaps even more surprising than the reorganization seen in the long term are the short-term changes in receptive field size and topography. Stimulus-dependent changes in receptive field size have been observed in the somatosensory cortex, where repeated stroking of a body part over a period of months leads to a decrease of receptive field size¹¹, in effect the opposite of the type of experiment done here. Our results suggest that the subthreshold influences from outside the classical receptive field can be quickly unmasked when the ascending input is inactivated, and these short term effects represent a surprising degree of plasticity of cortical topography and receptive field structure for adult animals. The immediate changes may represent the neural substrate of perceptual phenomena such as surface fill-in of colour and texture²⁵⁻²⁹, illusory contours³⁰, and learning

effects^{31,32}, and raises the possibility that dynamic changes in receptive field structure may occur continuously during normal vision. □

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1. Kalaska, J. & Pomeranz, B. *J. Neurophysiol.* **42**, 618-633 (1979).
2. Merzenich, M. M. et al. *J. comp. Neurol.* **224**, 591-605 (1984).
3. Clark, S. A., Allard, T., Jenkins, W. M. & Merzenich, M. M. *Nature* **332**, 444-445 (1988).
4. Sanes, J. N., Suner, S., Lando, J. F. & Donoghue, J. P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2003-2007 (1988).
5. Sanes, J. N., Suner, S. & Donoghue, J. P. *Expl Brain Res.* **79**, 479-491 (1990).
6. Robertson, D. & Irvine, D. R. F. *J. comp. Neurol.* **282**, 456-471 (1989).
7. Cusick, C. G., Wall, J. T., Whiting, J. H. Jr & Wiley, R. G. *Brain Res.* **537**, 355-358 (1990).
8. Gilbert, C. D., Hirsch, J. A. & Wiesel, T. N. in *Cold Spring Harbor Symp. quant. Biol.* **55**, 663-677 (1990).
9. Kaas, J. H. et al. *Science* **248**, 229-231 (1990).
10. Heinen, S. J. & Skavenski, A. A. *Expl Brain Res.* **83**, 670-674 (1991).
11. Jenkins, W. M., Merzenich, M. M., Ochs, M. T., Allard, T. & Guic-Robles, E. *J. Neurophysiol.* **63**, 82-104 (1990).
12. Calford, M. B. & Tweedale, R. *Nature* **332**, 446-448 (1988).
13. Donoghue, J. P., Suner, S. & Sanes, J. N. *Expl Brain Res.* **79**, 479-491 (1990).
14. Devor, M. & Wall, P. D. *Nature* **276**, 75-76 (1978).
15. Eysel, U. T., Gonzalez-Aguilar, F. & Mayer, U. *Expl Brain Res.* **41**, 256-263 (1981).
16. Gilbert, C. D. & Wiesel, T. N. *Nature* **280**, 120-125 (1979).
17. Gilbert, C. D. & Wiesel, T. N. *J. Neurosci.* **3**, 1116-1133 (1983).
18. Rockland, K. S. & Lund, J. S. *Brain Res.* **169**, 19-40 (1982).
19. Rockland, K. S. & Lund, J. S. *J. comp. Neurol.* **216**, 303-318 (1983).
20. Martin, K. A. C. & Whitteridge, D. *J. Physiol.* **353**, 463-504 (1984).
21. Gilbert, C. D. & Wiesel, T. N. *Vision Res.* **30**, 1689-1701 (1990).
22. Ts'o, D., Gilbert, C. & Wiesel, T. N. *J. Neurosci.* **6**, 1160-1170 (1986).
23. Ts'o, D. & Gilbert, C. *J. Neurosci.* **8**, 1712-1727 (1988).
24. Gilbert, C. D. & Wiesel, T. N. *J. Neurosci.* **9**, 2432-2442 (1989).
25. Yarbus, A. L. *Biophysics*, **2**, 683-690 (1957).
26. Krauskopf, J. *Am. J. Psychol.* **80**, 632-637 (1961).
27. Crane, H. D. & Piantanida, T. P. *Science* **221**, 1078-1079 (1983).
28. Ramachandran, V. S. & Gregory, R. L. *Nature* **350**, 699-702 (1991).
29. Paradiso, M. A. & Nakayama, K. *Vision Res.* **31**, 1221-1236 (1991).
30. Kanizsa, G. *Organization in Vision. Essays on Gestalt Perception* (Praeger, New York: 1979).
31. McKee, S. P. & Westheimer, G. *Perception & Psychophysics* **24**, 25-62 (1978).
32. Fendick, M. & Westheimer, G. *Vision Res.* **23**, 145-150 (1983).

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Genetic immunization is a simple method for eliciting an immune response

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To produce an immune reaction against a foreign protein usually requires purification of that protein, which is then injected into an animal. The isolation of enough pure protein is time-consuming and sometimes difficult. Here we report that such a response can also be elicited by introducing the gene encoding a protein directly into the skin of mice. This is achieved using a hand-held form of the biolistic system¹⁻⁴ which can propel DNA-coated gold microprojectiles directly into cells in the living animal^{3,5,6}. Genetic immunization may be time- and labour-saving in producing antibodies and may offer a unique method for vaccination.

Young (8-15 weeks old) mice were inoculated in the ear with microprojectiles coated with plasmids containing the genomic copy of the human growth hormone (hGH) gene under the transcriptional control of either the human β -actin promoter⁷ or the cytomegalovirus (CMV) promoter⁸. Production of antibody directed against hGH was monitored by assaying sera from tail-bleeds for the capacity to immunoprecipitate ¹²⁵I-labelled hGH. Figure 1 depicts the time-course of appearance

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TABLE 1 Number of genetically inoculated mice producing antibodies

Strain	Sex	Number of mice producing anti-hGH			Number of mice producing anti-hAAT	Number of mice producing both anti-hGH and anti-hAAT
		High* titre	Low† titre	None‡		
ICR	F	18/34	12/34	4/34	2/2	4/4
CFW	M	0/4	1/4	3/4	—	—
C57BL/6	F	2/2	0/2	0/2	—	—

Antibody responses to hGH after genetic inoculation of skin cells were determined by radioimmunoassay and/or western blot analysis as described in Figs 1 and 2a. Antibodies to hAAT were determined by western blot analysis as shown in Fig. 2b. Antibodies were detectable in most mice within 2 weeks of the primary inoculation. The four ICR mice and the three CFW mice that failed to respond to genetic immunization were killed about 1 month and 2 months after the primary inoculation, respectively. The sources of mice were: ICR (Harlan), CFW (Charles River) and C57BL/6 (Jackson).

* High titre, >0.5 ng hGH precipitable per μ l of serum about 1 month after the primary inoculation.

† Low titre, 0.02–0.5 ng hGH precipitable.

‡ None, <0.02 ng hGH precipitable.

of anti-hGH antibody in three individual mice representing high, medium and low responses. Table 1 shows that 88% (30/34) of ICR strain mice produced antibodies within a few weeks of inoculation. The CFW strain male mice seem less responsive to this approach than the ICR and C57BL/6 strain female mice tested. The antibody titres of mice showing responses to the hGH gene were arbitrarily grouped into high and low categories. Eighteen out of 34 ICR mice had antibodies capable of precipitating >0.5 ng hGH protein per microlitre of serum about one month after the primary inoculation. Nanograms of hGH protein could be detected in the genetically inoculated skin one day after inoculation⁵, but not in the blood of the treated mice.

Several lines of evidence support the conclusion that antibody is being produced against hGH. First, preimmune sera (Fig. 1) and sera from control mice inoculated with irrelevant plasmids (data not shown) precipitated background amounts of hGH. Second, the precipitation of labelled-hGH by the test sera was specifically competed by purified, unlabelled-hGH (data not

shown). Third, a 1:5,000 dilution of the test sera reacted in a western blot with purified hGH but not with other proteins (Fig. 2a).

We used the following protocol to investigate whether the primary response could be augmented. Three mice that had received a primary genetic inoculation and were producing anti-hGH antibody were inoculated again with the hGH plasmid. A fourth mouse that was also producing anti-hGH antibody was treated with a control plasmid expressing the firefly luciferase gene. There were variable but distinct responses to the hGH-DNA boost whereas the control boost showed little change in antibody levels (Fig. 3). Mice producing anti-hGH antibody showed no local inflammation when bombarded again in the ear with the hGH plasmid. We thus conclude that it may be possible to augment the immune response by subsequent DNA boosts. In addition to variations in the genetic background of individual mice, some of the variability in the level of anti-hGH produced by both primary and secondary treatments

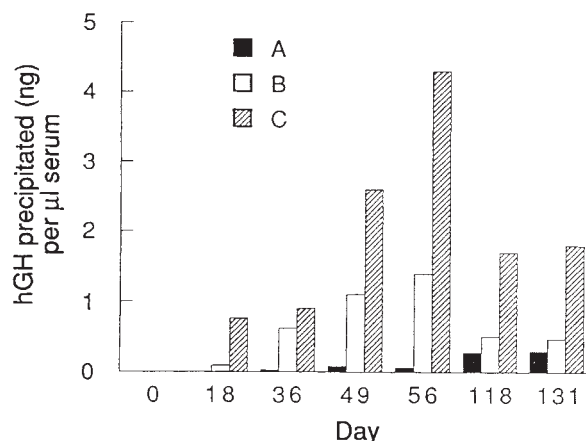


FIG. 1 Time course for the production of antibody to hGH in three mice (A, B, C) after genetic inoculation.

METHODS. Mice were inoculated in the skin of the ear with a plasmid expressing the hGH gene. Blood (50–100 μ l) was withdrawn from the tail on the days indicated. The amount of antibody to hGH, under linear assay conditions, was assayed by incubating 1 μ l diluted sera with 1 μ l ¹²⁵I-labelled hGH (DuPont-NEN, 84–88 μ Ci ml⁻¹; 113–116 μ Ci μ g⁻¹) for 1 h at room temperature. Protein A-agarose beads (4 μ l; Pierce) were added and the slurry incubated for 12–18 h at 4 °C. The beads were then pelleted by centrifugation and washed thoroughly with PBS before determining the c.p.m. retained. Sera from pre-genetic immunization and from mice inoculated with the luciferase gene precipitated an average of 2 pg ¹²⁵I-labelled hGH. Values were normalized to nanograms of hGH precipitated per μ l serum. Each data point represents the average of duplicate samples. All animals were treated in accordance with an approved institutional protocol as detailed in ref. 5.

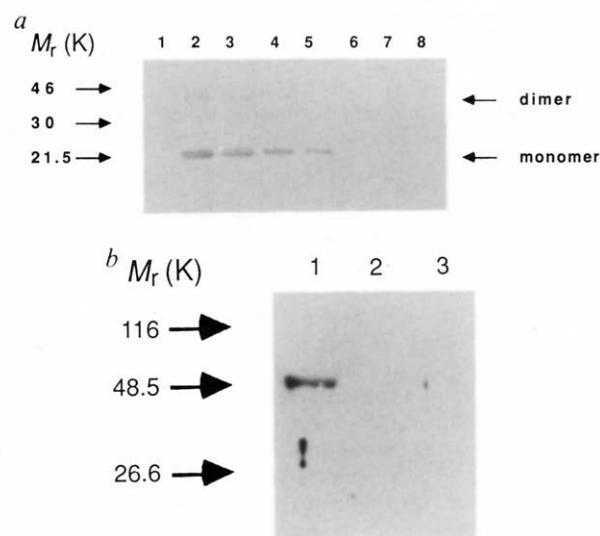


FIG. 2 a, Detection of antibodies against hGH in the sera of genetically immunized mice by western blot analysis. Serum from a genetically immunized animal (118 days after the primary inoculation) was diluted 1:5,000 and reacted with purified hGH protein (CalBiochem) that had been separated in a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Lane 1, 1 μ g luciferase protein; lanes 2–7: hGH, 1 μ g (lane 2), 0.5 μ g (lane 3), 0.25 μ g (lane 4), 0.125 μ g (lane 5), 0.0625 μ g (lane 6), 0.0312 μ g (lane 7); lane 8, 1 μ g BSA. b, Detection of antibodies against hAAT. Serum from an immunized mouse (25 days after the primary inoculation) was diluted 1:625 and reacted with purified hAAT protein (CalBiochem) which had been separated in a 10% SDS-polyacrylamide gel and transferred as in a. Lane 1, 1 μ g hAAT protein; lane 2, 1 μ g hGH protein; lane 3, 1 μ g BSA.

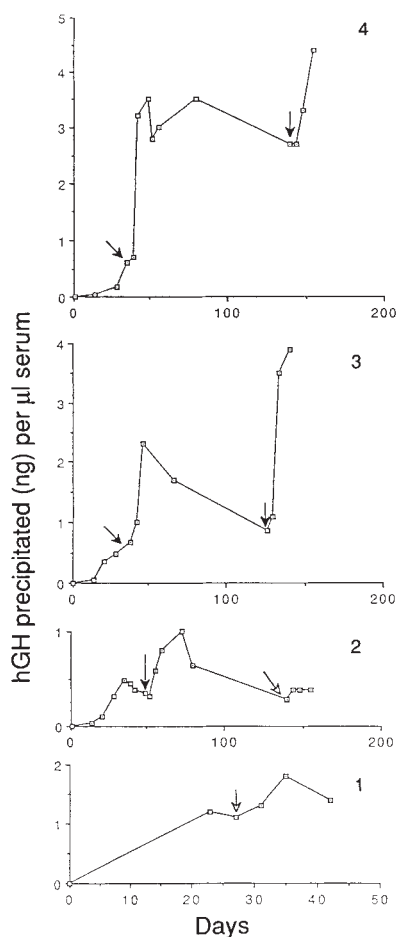


FIG. 3 Effects of genetic immunization boosts on anti-hGH antibody production. Four mice producing antibodies against hGH after a primary treatment at day 0 were inoculated again at different times with either the hGH plasmid (solid arrows) or the luciferase plasmid (open arrows). Three of the mice were inoculated a third time with either the luciferase plasmid (number 2) or the hGH plasmid (numbers 3 and 4). The sera were assayed as described in Fig. 1. The line between the first and second data points for number 1 is included for visualization.

probably arises from the operation of the device or the coating of the DNA onto the microprojectiles because treatment of skin cells with a luciferase reporter plasmid also leads to variable levels of expression^{5,6}.

We have tested whether this technique might be generally applicable to other proteins by genetically inoculating with a plasmid containing the complementary DNA copy of the human α 1-antitrypsin (hAAT) gene. This gene was also expressed from the CMV promoter and introduced into the skin of the ear. All the mice inoculated produced antibody to hAAT (6/6; Table 1) as determined by western blot analysis (Fig. 2b). Mice genetically immunized with both hAAT and hGH at the same time produced antibody to both proteins (4/4). That the two mice inoculated with only the hAAT plasmid produced anti-hAAT antibody demonstrates that the pharmaceutical effect of hGH is not required to elicit the response. Our results are from inoculations with the hand-held biolistic device. But a simple adaption⁶ of the commercially available biolistic instrument (BioRad) can also be used for genetic immunization. By contrast, injection of the hGH plasmid (50 μ g) into the skin of two mice with a hypodermic needle did not produce a response. Biolistic inoculations into the liver produced hGH⁵ but did not elicit an immune reaction (eight mice tested).

This technique may have at least two useful applications. One is to simplify the procedure and shorten the time required to

produce antibodies to particular proteins by eliminating the steps for protein purification and adjuvant administration. The second, more speculative, is the genetic vaccination of animals against pathogenic infection by producing foreign antigens in restricted subsets of self-cells that mimics natural infections. In this regard, differences in immunological response between genetic and conventional immunization (for example, the duration and magnitude of antibody production or level of T-cell response) may give this protocol useful features. The ability to raise antibodies to both hGH and hAAT indicates that this technique may generally be applicable to secreted proteins. Whether it will be effective with nonsecreted proteins remains to be seen. This technique offers a simple method to elicit antibodies to some proteins and may provide a tool for manipulating the immune response. □

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1. Sanford, J. C., Klein, T. M., Wolf, E. D. & Allen, N. *Particulate Sci. Technol.* **5**, 27-37 (1987).
2. Armaleo, D. et al. *Curr. Genet.* **17**, 97-103 (1990).
3. Sanford, J. C. et al. *Technique* **3**, 3-16 (1991).
4. Sanford, J. C., Smith, R. D. & Russell, J. A. *Meth. Enzym.* (in the press).
5. Williams, R. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2726-2730 (1991).
6. Johnston, S. A. et al. *In Vitro cell. dev. biol.* **27**, 11-14 (1991).
7. Leavitt, J. et al. *Molec. cell. Biol.* **4**, 1961-1969 (1984).
8. Boshart, M. et al. *Cell* **41**, 521-530 (1985).

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Targeted disruption of μ chain membrane exon causes loss of heavy-chain allelic exclusion

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BURNET'S clonal selection theory¹ suggests that each B lymphocyte is committed to a single antibody specificity. This is achieved by a programme of somatic rearrangements of the gene segments encoding antibody variable (V) regions, in the course of B-cell development^{2,3}. Evidence from immunoglobulin-transgenic mice and immunoglobulin-gene-transfected transformed pre-B cells suggests that the membrane form of the immunoglobulin heavy (H) chain of class μ (μ m), expressed from a rearranged H-chain (IgH) locus, may signal allelic exclusion of the homologous IgH locus in the cell⁴⁻⁶ and initiation of light (L)-chain gene rearrangement in the Ig κ loci⁶. We report here that targeted disruption of the membrane exon of the μ chain indeed results in the loss of H-chain allelic exclusion. But, some κ chain gene rearrangement is still observed in the absence of μ m expression.

The μ MT mouse mutant was generated by targeted disruption of the membrane exon of the μ chain by a neomycin resistance gene in embryonic stem cells, and then introduction of the mutation into the mouse germ line⁷. Homozygous mutant mice cannot produce B cells because B-cell development is arrested at the stage of pre-B cells. But in heterozygous mutant mice normal numbers of B cells are produced. These cells express surface-bound immunoglobulin molecules whose μ chains are encoded by the wild-type IgH locus⁷. This allows a direct test of whether μ m expression is required for allelic exclusion at the H chain locus. The first gene rearrangement occurring in the course of B-cell development is the joining of D_H- and

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TABLE 1 The $C\mu$ locus bearing the μ MT mutation fails to signal allelic exclusion

Genotype	B cells expressing IgH ^a and IgH ^b expected (%)	LPS-blasts expressing $c\mu^a$ and $c\mu^b$ (%) [*]		
		Spleen	Bone marrow	
			Total	Newly generated B cells
IgH ^a , μ MT/IgH ^b , +	12.2	5.7 6.1	9.9 6.8	20 [†] 25 [‡]
IgH ^a , +/IgH ^b , +	0?	0.3 0.3	<0.1	ND

^{*} Spleen or bone marrow cells (10^6 ml^{-1}) from heterozygous mutant mice were cultured in CG medium (Cameron) supplemented with $50 \mu\text{g LPS ml}^{-1}$ for 5 days. Cytospin slides were then prepared and the cells were fixed and stained first with biotin-conjugated monoclonal antibody (MoAb) MB86 (ref. 23) (anti- μ^b) and then with fluorescein isothiocyanate (FITC)-conjugated MoAb RS3.1 (ref. 24) (anti- μ^a) and Texas Red-conjugated streptavidin. Each number represents the frequency of double-positive cells among the positive cells in the analysis using individual mice.

[†] Bone marrow cells were stained with phycoerythrin (PE)-conjugated MoAb RA3-6B2 (ref. 25) (anti-CD45R(B220)) and FITC-conjugated MoAb R33-24-12 (ref. 26) (anti- μ). Newly generated B cells (CD45R^{dim}, μ^+ cells in the lymphocyte gate) were sorted by FACStar plus (Becton Dickinson).

[‡] B cells were generated from a clonal progenitor obtained from a limiting dilution assay of the bone marrow cells on the PA6 stromal layer with IL-7 as described before²⁷. Cells (2×10^4) were cultured on a BALB/c 3T3 fibroblast-feeder layer in 600 μl CG medium supplemented with 5% FCS and $50 \mu\text{g LPS ml}^{-1}$ for 5 days as described before²⁸.

ND, not determined.

J_H -gene segments on both homologous chromosomes, generating a pro-B cell³. If, in the μ MT/+ mice, a pro-B cell undergoes subsequent V_H - D_HJ_H joining in the mutated IgH locus first and the joining is in frame ('productive'), the resulting pre-B cell can express a μ chain of the secreted form (μ s), but cannot express μ m. If μ m expression is required for allelic exclusion, then such a cell would still be able to undergo V_H - D_HJ_H joining in the wild-type IgH locus; and if this second rearrangement is also productive, the cell would express two different μ chains, one of which could be membrane-bound.

Assuming that the frequency of productive $V_HD_HJ_H$ joints is 1/3 and that the cell has an equal chance to undergo the first V_H - D_HJ_H joining on either of the two homologous chromosomes, the expected frequency of double producers in the B-cell population would be 16.7%. This value has to be corrected to 12.2% because 80% of D_H segments joined to J_H segments in reading frame 3 carry stop codons^{8,9}.

For the determination of double producing cells in μ MT/+ mice we made use of the fact that these animals carry a μ gene of the *a* allele (μ^a) in the mutated locus, whereas the wild-type μ gene is of allotype *b* (ref. 7). Spleen cells from μ MT/+ animals and (BALB/c (IgH^a) $\times$ C57BL/6(IgH^b)F₁ mice were isolated and activated *in vitro* by bacterial lipopolysaccharide (LPS). The activated B-cell blasts were screened for the expression of cytoplasmic IgM of *a* and *b* allotype using fluorescence micro-

scopy (Table 1). The frequency of double producers was close to 6% in the μ MT/+ mice, whereas such cells were scarcely detectable in the controls. Double producers were thus indeed present in the splenic B cells from heterozygous mutant mice, but less abundant than theoretically expected. This might reflect counterselection of such cells when they expand in the peripheral immune system¹⁰. In accord with this, we found low and variable amounts of IgM antibodies of *a* allotype in the sera of μ MT/+ mice, ranging from <1–7% of the total IgM. Low levels (0.3–1%) of IgG1^a were also detectable, as expected because switching is often concomitantly induced on both IgH loci of a B cell¹¹ (data not shown). To minimize possible effects of cellular selection, we also determined double producing cells in the microenvironment where B cells are generated from progenitor cells, namely the bone marrow. There was a great increase in the frequency of the double producing cells when newly generated bone marrow B cells were analysed, isolated either *ex vivo* by fluorescence-activated cell sorting (FACS) or generated *in vitro* from B-cell progenitors (Table 1). The frequencies were now greatly above the theoretical expectation, reaching values of up to 25%. Because V_H to D_HJ_H rearrangements are controlled by the IgH intron enhancer¹², we speculate that this effect might be due to the neomycin resistance gene in the targeted IgH locus. Indeed, if all B-cell progenitors in the μ MT/+ mice undergo V_H to D_HJ_H rearrangement on the mutated

FIG. 1 Quantitative PCR analysis of V_K - J_K rearrangements. Serially diluted genomic DNA extracted from bone marrow cells of the mice (3 months old) of listed genotype or genomic DNA (50 ng) from embryonic stem cells (ES) was subjected to PCR. We used degenerate primers hybridizing to most V_K segments and a J_K2 primer as described¹⁵. Fragments of about 500 and 130 bp were amplified for V_KJ_K1 and V_KJ_K2 rearrangements, respectively (see lower part of fig.). To control the amplification, a standard plasmid in which a cloned V_K segment ($V_KA20/44$ (ref. 34)) was joined to the genomic J_K sequence upstream of J_K1 was added to each reaction (10 fg per tube). The standard plasmid gives rise to a 570-bp fragment in the gene amplification reaction. The latter was done in 50 μl 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 2 mM MgCl_2 , 0.1 μM primers, 0.4 mM dNTPs and 2 U *Taq* polymerase for 40 cycles (1.5 min at 94 °C and 2.0 min at 72 °C). After completion of the reaction, 10 μl samples were run on a 2% agarose gel. The upper panel shows the gel after staining with ethidium bromide. The identity of the amplified bands was confirmed by Southern blotting using a J_K1 -3 genomic fragment as a probe (data not shown).

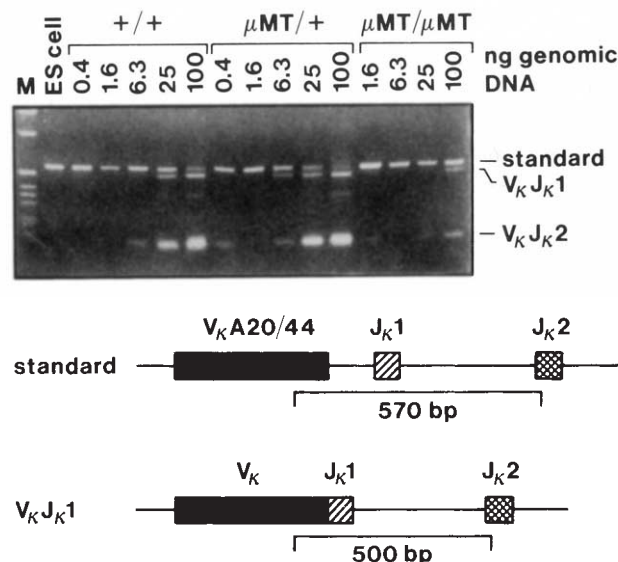


TABLE 2 $V_H D_H J_H$ junctions of the mutated (Igh^a) allele in amplified cDNA libraries from Igh^a , $\mu MT/Igh^b$, + mice

	Spleen δ^{a+} δ^{b+} B cells	Bone marrow $\mu^+ \delta^-$ B cells
Productive	17	5
Non-productive	1	1
$V_H D_H J_H^a/V_H D_H J_H^b$	80/97	78/181

Spleen cells from 2-month-old mice were stained with biotinylated MoAb 4/4D7 (anti- δ^b ; gift of T. Tokuhisa) and FITC-conjugated MoAb 10-4-12 (ref. 29) (anti- δ^a), followed by PE-streptavidin. δ^{a+} , δ^{b+} cells in the lymphocyte gate (about 3% of the total δ^+ cells) were isolated by FACS. Bone marrow μ^+ , CD45R(B220)^{dim} B cells (which should be δ^- (ref. 30) were also isolated. No contaminating plasma cells were found by fluorescence microscopy in this fraction. Polymerase chain reaction (PCR)-mediated cDNA libraries were made using each sorted fraction of cells as described before³¹. The 5' primer was specific for the $V_H 7183$ family (5'-GCAAGCTTGGTGGAGTCTGGGGA-3') and the 3' primer for the $C\mu$ exon 2 (5'-CAGGATCCCGTGGTGGGACGAA-3'). Bacterial colonies were screened with a $C\mu^a$ -specific oligonucleotide (5'-GCAGATCTCTGTTTTC-3', the one base difference from $C\mu^b$ is underlined³²) by colony hybridization, and $V_H D_H J_H$ junctions of randomly selected clones were sequenced using a $C\mu 2$ primer³¹ as described before³³. Twelve of these clones were also sequenced using the $C\mu$ exon 2 primer (see above) and all contain $C\mu^a$ sequences. Shown are numbers of clones containing productive (upper) or non-productive (middle) $V_H D_H J_H$ joints, and the number of colonies hybridizing or not hybridizing with the $C\mu^a$ oligo-probe (bottom).

chromosome first, the expected frequency of double producers would be 24.4%, close to the experimentally observed values (Table 1).

To demonstrate the presence of productive $V_H D_H J_H$ rearrangements in the mutated IgH locus of the heterozygous mutant mice at the molecular level, we amplified and sequenced $V_H D_H J_H$ joints of *a* and *b* allotype from complementary DNA of B cells from these animals. Two types of B cells were used in this analysis. The first were splenic B cells expressing IgD of both allotypes on the surface. Such cells are detectable in the animals in small amounts⁷ (3–4%; data not shown) and can be isolated by cell sorting. The second B-cell population were immature ($IgM^+ IgD^-$) bone marrow B cells. Results are summarized in Table 2. Productive $V_H D_H J_H$ joints from the mutated (*a* allotype) IgH locus, mostly containing N sequences (not encoded in germ line) at the recombination breakpoints (not shown), could indeed be isolated from both cell populations. From the IgD double producers equal numbers of rearrangements were isolated from the mutated and the wild-type locus, underlining the purity of the sorted population. In the newly generated bone marrow B cells, 30% of the joints came from the mutated locus. Most of these joints were productive, consistent with transcripts from productive joints being intracellularly more stable than transcripts from non-productive ones¹³. The results thus agree well with the frequency of double producers in this cell population.

In addition to signalling allelic exclusion at the IgH locus, the μm chain has been suspected to be involved in the initiation of $V_\kappa-J_\kappa$ joining, in the frame of the ordered programme of V-region gene rearrangements in B-cell development⁶, although the evidence is controversial¹⁴. If a signal by μm is essential for $V_\kappa-J_\kappa$ joining, then no such event should be seen in mutant mice carrying the μMT mutation on both IgH loci. We investigated this by amplifying $V_\kappa J_\kappa$ joints from bone marrow cells of $\mu MT/\mu MT$ animals and heterozygous mutant mice for control, using appropriate oligonucleotide primers for specific gene amplification¹⁵ and an internal control to allow quantification. The results demonstrate the presence of $V_\kappa J_\kappa$ joints in the bone marrow of the homozygous mutant mice (Fig. 1). The frequency of these joints is about 1/20 compared with the control bone

marrow. Rearrangements were not seen in DNA prepared from kidney or liver of the mutant animals (data not shown).

Our analysis demonstrated that targeted disruption of the membrane exon of the μ chain results in allelic inclusion at the IgH locus. This implies that μm expression is an essential step in the control of H chain allelic exclusion, as had been suggested^{4–6}. Formally, we cannot exclude that the IgH locus bearing the μMT mutation is itself not subject to allelic exclusion by the wild-type allele, owing to transcriptional activation near the inserted neomycin gene. But that the productively rearranged IgH locus is efficiently transcribed in allelically excluded B cells argues against a control of allelic exclusion at the level of $V_H D_H J_H$ or $D_H J_H$ transcription. Experimental evidence has identified the 'accessibility' of unrearranged V_H genes as the likely target of control¹².

B cells producing H chains from both endogenous IgH loci were readily detectable in the heterozygous mutant mice. The absence of such cells in wild-type animals (Table 1) is thus not a technical artefact¹⁶ but contradicts the 'stochastic' allelic exclusion model of Cohn and Langman¹⁶ which predicts double producing cells at a frequency of around 10%. In the 'regulated' model of allelic exclusion³ the product of a productive H chain gene rearrangement, that is, the μ chain (supposedly in its membrane form^{4–6}), stops further rearrangements in the IgH loci and initiates L-chain gene rearrangements. In support of this model, μm is expressed in pre-B cells in association with the 'surrogate' L chains $\lambda 5$ and $VpreB$ before L-chain gene rearrangement^{17–21}, and the analysis of the μMT mouse mutant has shown that its expression is critical both for B-cell development at this stage⁷ and for H-chain allelic exclusion (this paper). But as the present data demonstrate, some κ chain rearrangements also occur in the absence of μm , in accord with earlier work in humans demonstrating immunoglobulin gene rearrangements in Ig κ but not IgH loci of a fraction of Epstein-Barr virus-transformed B-cell progenitors²². Whether this reflects a general, perhaps minor 'leakiness' of the programme of ordered gene rearrangements or the properties of a subset of B cells remains to be elucidated. □

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1. Burnet, F. M. *The Clonal Selection Theory of Acquired Immunity* (Cambridge Univ. Press, 1959).
2. Tonegawa, S. *Nature* **302**, 79–84 (1983).
3. Alt, F. W. *et al. EMBO J.* **3**, 1209–1219 (1984).
4. Nussenzweig, M. C. *et al. Science* **236**, 816–819 (1987).
5. Manz, J., Denis, K., Witte, O., Brinster, R. & Storb, U. *J. exp. Med.* **168**, 1363–1381 (1988).
6. Reth, M., Petrac, E., Wiese, P., Lobel, L. & Alt, F. W. *EMBO J.* **6**, 3299–3305 (1987).
7. Kitamura, D., Roes, J., Kühn, R. & Rajewsky, K. *Nature* **350**, 423–426 (1991).
8. Ichihara, Y., Hayashida, H., Miyazawa, S. & Kurosawa, Y. *Eur. J. Immun.* **19**, 1847–1854 (1989).
9. Gu, H., Kitamura, D. & Rajewsky, K. *Cell* **65**, 47–54 (1991).
10. Köhler, G., *Proc. natn. Acad. Sci. U.S.A.* **77**, 2197–2199 (1980).
11. Radbruch, A., Müller, W. & Rajewsky, K. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3954–3957 (1986).
12. Ferrier, P. *et al. EMBO J.* **9**, 117–125 (1990).
13. Jäck, H. M., Berg, J. & Wabl, M. *Eur. J. Immun.* **19**, 843–847 (1989).
14. Blackwell, T. K. *et al. EMBO J.* **8**, 735–742 (1989).
15. Schlissel, M. S. & Baltimore, D. *Cell* **58**, 1001–1007 (1989).
16. Cohn, M. & Langman, R. E. *Immun. Rev.* **115**, 7–142 (1990).
17. Pillai, S. & Baltimore, D. *Curr. Topics Microbiol. Immun.* **137**, 136–139 (1988).
18. Kerr, W. G. *et al. Int. Immun.* **1**, 355–361 (1989).
19. Karasuyama, H., Kudo, A. & Melchers, F. *J. exp. Med.* **172**, 962–972 (1990).
20. Tsubata, T. & Reth, M. *J. exp. Med.* **172**, 973–976 (1990).
21. Cherayil, B. J. & Pillai, S. *J. exp. Med.* **173**, 111–116 (1991).
22. Kubagawa, H., Cooper, M. D., Carroll, A. J. & Burrows, P. D. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2356–2360 (1989).
23. Nishikawa, S., Sasaki, Y., Kina, T., Amagi, T. & Katsura, Y. *Immunogenetics* **23**, 137–139 (1986).
24. Schüppel, R., Wilke, J. & Weiler, E. *Eur. J. Immun.* **17**, 739–741 (1987).
25. Coffman, R. L. *Immun. Rev.* **69**, 5–23 (1982).
26. Grützmann, R. thesis, Univ. Cologne (1981).
27. Hayashi, S. *et al. J. exp. Med.* **171**, 1683–1695 (1990).
28. McHeyzer-Williams, M. G. & Nossal, G. J. V. *J. Immun. Meth.* **119**, 9–17 (1989).
29. Oi, V. T. & Herzenberg, L. A. *Molec. Immun.* **16**, 1005–1017 (1979).
30. Förster, I., Vieira, P. & Rajewsky, K. *Int. Immun.* **1**, 321–331 (1989).
31. Gu, H., Förster, I. & Rajewsky, K. *EMBO J.* **9**, 2133–2410 (1990).
32. Gause, A., Yoshida, N., Kappen, C. & Rajewsky, K. *Eur. J. Immun.* **17**, 981–990 (1987).
33. Weiss, U. & Rajewsky, K. *J. exp. Med.* **172**, 1681–1689 (1990).
34. Kocks, C. & Rajewsky, K. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8206–8210 (1988).

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Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions

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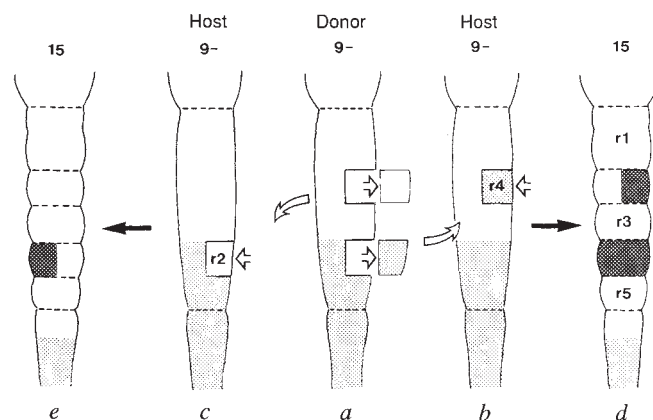


FIG. 1 Diagram of rhombomere transplantation experiment. At stage 9—the hindbrain is a tapered cylinder with the r5/6 boundary evident but the r3/4 and r4/5 boundaries (dotted lines) not yet formed. The region of low-level *Hox-2.9* expression is shaded (a–c). The presumptive r4 region, determined by measurement along the AP axis (a), was removed and inserted into r2 position in an isochronic host embryo (b), maintaining polarity. Alternatively, the presumptive r2 region (a) was grafted in place of r4 (c). Donor neuroepithelial tissue was grafted either together with adherent mesoderm cells, or after their enzymatic removal. Embryos were then incubated to stage 15–19 (d, e). Rhombomere 4 later expresses *Hox-2.9* at high level, both in normal and ectopic positions (d). Rhombomere 2 does not express *Hox-2.9* even when grafted to the r4 position (c, e).

METHODS. Rhombomeres were excised unilaterally from stage 9– to 10+ donor embryos using flame-sharpened needles of 100- μ m tungsten wire. Grafted pieces included neuroepithelium, surface ectoderm and mesoderm. In grafts made without mesoderm, transverse block sections of the body axis, one rhombomere thick, were removed from the appropriate level and immersed in 1 mg ml⁻¹ dispase (Boehringer) for 5 min, after which the neuroepithelium could easily be separated from surrounding tissues. Rhombomeres were then washed repeatedly in Howard's Ringer containing 20% fetal calf serum. Host embryos were prepared by sub-blastodermal injection of India ink and unilateral removal of the presumptive r2 (r4) region *in ovo*. Rhombomeres were transferred by pipette and manoeuvred into place, maintaining their normal polarity. Operated eggs were sealed with tape and returned to the incubator for 24–36 h. Embryos were then removed, and those in which the graft had integrated were fixed in 4% formaldehyde and processed for *in situ* hybridization.

FIG. 2 *In situ* hybridizations showing pattern of *Hox-2.9* expression in embryos with transplanted rhombomeres. a, High-power dark-field micrograph at stage 11. Low-level expression is continuous from the r3/4 boundary caudally. The level is already higher in r4 by this stage. b, Low-power bright-field micrograph at stage 15. Individual rhombomeres can be identified by their proximity to the otic vesicle (ov). c–h, High-power dark-field micrographs; arrow heads indicate position of grafted tissue. c, By stage 15, the level of expression in r4 is considerably higher than in more caudal regions. Grafts of r4 to the r2 position at stage 9– (d) and 9+ (e) consistently express *Hox-2.9* at similar levels to the normal r4 when analysed at stage 15 (d) or 19 (e). f, Grafts made at the same early stage but where the pial mesenchyme was removed by earlier enzyme treatment also showed high-level *Hox-2.9* expression at stage 15. g, h, Reciprocal grafts of r2 to the r4 position at stage 9+, either with mesenchyme attached (g) or after its removal (h), showed no expression of *Hox-2.9* at stage 17. All micrographs are of horizontal sections. All operated embryos analysed by *in situ* hybridization displayed either a *Hox-2.9*-positive ectopic r4 ($n=14$; 6 with mesenchyme, 8 without) or a *Hox-2.9*-negative ectopic r2 ($n=5$).

METHODS. The *Hox-2.9* probe was a subcloned 1-kilobase *Xho*-*Pst* fragment in the vector pSP65 which was isolated from a cosmid containing three genes in the chicken *Hox-2* complex. This probe spans the homeobox and 3' untranslated region and the gene is identical to *Ghox-lab* based on

sequence of the homeobox¹⁴. ³⁵S-labelled antisense probe was synthesized by SP6 transcription and *in situ* hybridization was done as described previously²⁶ except that the probes were resuspended to a final activity of 1×10^4 c.p.m./ μ l⁻¹. No signals were detected with sense control probes.

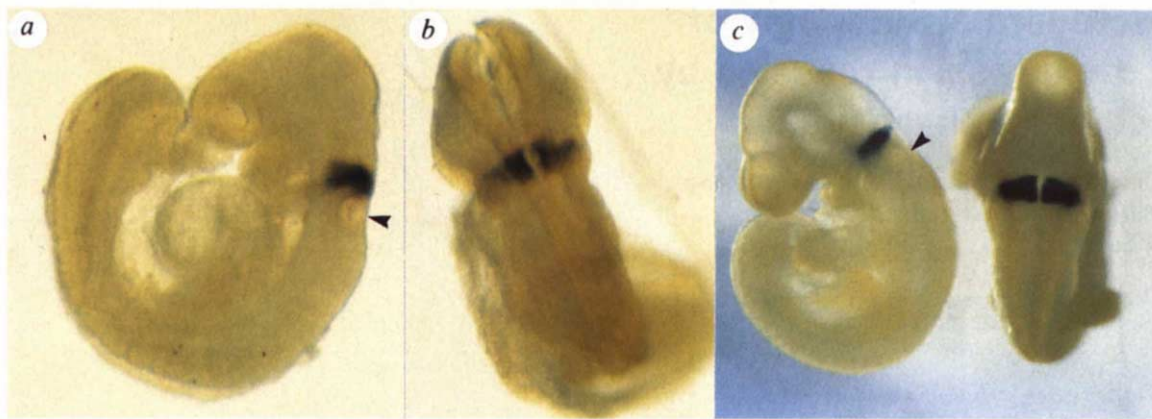


FIG. 3 Independent regulation of the early and late phase of *Hox-2.9* expression. Transgenic mice were generated with a *Hox-2.9-lacZ* fusion construct which reproduces only the r4-restricted pattern of *Hox-2.9* expression. Expression of the transgene is first observed in a few cells of the future r4 region at 8.25 d.p.c., and expression then increases after the late pattern of the endogenous gene. *a*, *b*, Lateral and dorsal views of a 8.5 d.p.c. and *c*, a 9.5 d.p.c. transgenic embryo stained for β -galactosidase activity. Note that the transgene is only expressed in the developing r4 region and migrating neural crest cells, when the endogenous gene is also

highly expressed in other regions of the embryo at 8.5 d.p.c.^{12,23}. The arrows indicate the position of the otic vesicle.

METHODS. Transgenic mice were produced by microinjection into fertilized eggs from F₁ backcrosses (CBA $\times$ C57) and transfer to pseudopregnant females. The DNA construct was a genomic fragment of the mouse *Hox-2.9* gene into which the *Escherichia coli* β -galactosidase reporter gene was inserted to create a fusion protein. Transgenic embryos were identified by polymerase chain reaction (PCR) analysis and stained for β -galactosidase activity in whole-mount preparations as described previously²⁷.

both of position in the neuroepithelium and of signals from the underlying mesoderm¹⁵.

We isolated a DNA probe for chick *Hox-2.9* and used it to follow the development of rhombomere 4 (r4), the single segment in which *Hox-2.9* is expressed at high levels^{11,12,14}. In presomite and early somite stages of both mouse^{11,17} and chick^{14,16}, *Hox-2.9* is uniformly expressed at low levels in the neural tube, up to an anterior limit that maps close to the future r3/4 boundary. Later, expression is no longer detectable in the region of r5 and r6 and there is a dramatic increase of transcripts in r4 alone (Figs 1 and 2*a-c*). Transgenic experiments in the mouse have shown that this high level of *Hox-2.9* expression, which is first detectable at the 5–6 somite stage (8.25 days post

coitum (d.p.c.), is mediated by a regulatory region ('stripe element') distinct from that responsible for the early domain of expression (Fig. 3; R.K. and H.M., unpublished data).

The presumptive r4 region was transplanted from donor embryos either before or at the time when its boundaries are being defined (at stage 9– to 10+, 6–11 somites). Grafts were made homolaterally, replacing the presumptive r2 of an isochronic host embryo. In reciprocal grafts, the presumptive r2 was grafted in place of r4. Thus the region that would normally later express *Hox-2.9* at high level was moved to a position where the gene is not expressed, and vice versa (Fig. 1).

In all cases, *in situ* hybridization of operated embryos at stages 15–19 showed a normal pattern of *Hox-2.9* expression in

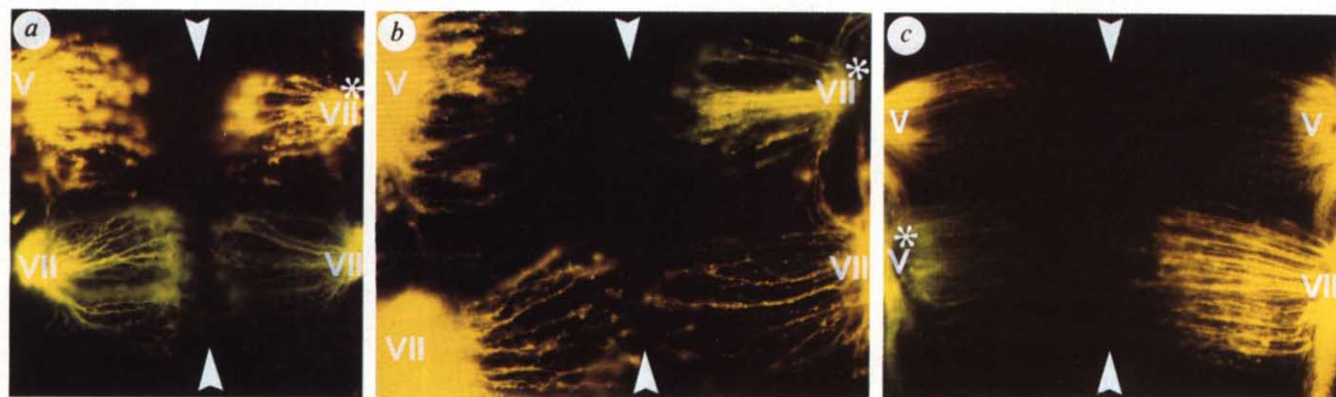


FIG. 4 Retrograde labelling of branchiomotor nerves in embryos with r4 transplanted in r2 position. The trigeminal motor nucleus (V) lies in r2 and r3 in normal chick embryos, whereas the facial motor nucleus (VII) lies in r4 and r5 (ref. 6). *, Motor nuclei in transplanted rhombomeres. Arrow heads show the midline. *a*, r4 to r2 graft at stage 9– with mesenchyme. Left, unoperated side of embryo at stage 17 shows normal pattern of trigeminal motor nucleus (yellow) and facial motor nucleus (green). Right, operated side shows how nucleus in ectopic r4 in trigeminal position (yellow) has morphology more closely similar to facial nucleus on control side, with a fan-shaped axon array and more medial cell bodies. *b*, r4 to r2 graft at stage 9– without mesenchyme. Left, unoperated side at stage 17 shows normal trigeminal motor nucleus (yellow) and normal facial nucleus (yellow). Right,

additional facial nucleus (green). *c*, r2 to r4 graft at stage 9–, without mesenchyme. Right normal trigeminal and facial nuclei, unoperated side of embryo (yellow) at stage 17. Left, trigeminal nucleus (green).

METHODS. For retrograde labelling of cranial nerves, embryos were fixed for 2 h in 4% formaldehyde, and dissected from the ventral side to expose the cranial nerves. The fluorescent dyes Dil and DiO (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate and 3,3'-dioctadecyloxycarbocyanine perchlorate; Molecular Probes) at 3 mg ml⁻¹ in 1:1 dimethylformamide and ethanol were injected by pressure using glass micropipettes. Embryos were left for 24 h in the dark at room temperature and then hindbrains were dissected out, mounted flat and viewed under green (Dil) or blue (DiO and Dil) fluorescence excitation.

the host r4. Embryos which had received a donor presumptive r4 in r2 position had an additional anterior stripe of *Hox-2.9* expression (Fig. 2*d-f*). Comparisons of signal intensity suggest that the level of expression in the ectopic r4 was equivalent to that in the host r4. The gene was upregulated irrespective of whether surrounding mesoderm cells were included in the graft, or were enzymatically removed before grafting. By contrast, transplants of r2 to the r4 position never expressed *Hox-2.9* (Fig. 2*g, h*), even when (grafted without mesoderm) they lay in direct contact with host mesoderm cells at r4 level. This indicates that the r4-restricted expression is a regional property of the neuroectoderm and that the underlying mesoderm does not regulate expression at these stages of development (compare with ref. 15).

Segment phenotype was examined in transplanted embryos by retrograde tracing of efferent cranial nerves to visualize the disposition of branchiomotor neurons. The motor neurons in r4 (facial nerve, VII) can be distinguished from those in r2 (trigeminal nerve, V) by virtue of the fan-shaped array of axons and the more medial position of cell bodies contributing to the facial nucleus. In transpositions of presumptive r4 to r2, retrograde labelling revealed that the facial motor nucleus had developed in the trigeminal position, irrespective of whether the grafted tissue included mesoderm cells (Fig. 4*a, b*). Similarly, reciprocal transpositions of presumptive r2 to r4 had the morphology of the trigeminal nucleus (Fig. 4*c*).

Our results show that the establishment of positional identity is intrinsic to the hindbrain from an early stage of development, immediately preceding segmentation. What role, if any, does the mesoderm have in this process? An argument for 'position-specific determination' of the neuroectoderm by the mesoderm, has been made on the basis of expression of *Hox-2.9* in the neuroepithelium of 7.75–8 d.p.c. mouse embryos being preceded by a coextensive domain of expression in the mesoderm¹⁵. Classic studies on amphibia in which different anteroposterior (AP) regions of mesoderm induced nervous tissue with different regional characters^{18,19} have recently received support from experiments using molecular markers^{20,21}. However, at least some aspects of AP pattern in the amphibian neural plate may emerge in exogastrulae, presumably without direct contact with the mesoderm²² (A. Ruiz i Altaba and T. M. Jessel, personal communication).

Our data show that as early as six somites, the hindbrain has a distinct regional identity. The segments are irreversibly committed at the time at which lineage compartments^{6,8} are formed; thereafter, rhombomere phenotype is maintained in a segment-autonomous manner and *Hox-2.9* is upregulated independently of AP position in the neuroepithelium and of the mesodermal environment of r4. These findings are consistent with *Hox-2.9* encoding the positional identity of r4. The independence of the regulatory elements that drive low- and high-level *Hox-2.9* expression in the neuroepithelium also allows the possibility of a wider role for *Hox-2.9* during hindbrain development. *Hox-1.6*, the paralogue of *Hox-2.9*, has an identical early domain of expression but lacks the high-level r4-restricted expression²³. In null mutations of *Hox-1.6* in mice^{24,25} there were early alterations to hindbrain segmentation²⁵ and both certain central nuclei and sensory ganglia of the posterior myelencephalon (r4–r7) were absent in later embryos^{24,25}. The briefly transient expression of *Hox-1.6* and low-level *Hox-2.9* in the posterior myelencephalon (between 8 and 8.5 d.p.c. in mouse²³, equivalent to 3–6-somite chick) suggests the involvement of both genes in defining this broad region, the anteriormost part of which (r4) is subsequently refined by high-level expression of *Hox-2.9*. The observation that in *Hox-2.9-lacZ* transgenic mice, the stripe element is active at the six-somite stage (Fig. 3), around the time of r4 boundary formation in the chick embryo is consistent with the idea that high-level *Hox-2.9* expression could be responsible for determining the positional identity of r4 alone. □

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- Graham, A., Papalopulu, N. & Krumlauf, R. *Cell* **57**, 367–378 (1989).
- Duboule, D. & Dolle, P. *EMBO J.* **8**, 1497–1505 (1989).
- Lewis, E. B. *Nature* **276**, 565–570 (1978).
- Harding, K. et al. *Science* **229**, 1236–1242 (1985).
- Akam, M. *Cell* **57**, 347–349 (1989).
- Fraser, S., Keynes, R. & Lumsden, A. *Nature* **344**, 431–435 (1990).
- Gräper, L. *Arch. mikrosk. Anat. Entw. Mech.* **83**, 371–426 (1913).
- Lumsden, A. *Trends Neurosci.* **13**, 329–335 (1990).
- Lumsden, A. & Keynes, R. *Nature* **337**, 424–428 (1989).
- Gaunt, S. J. *Development* **101**, 51–60 (1987).
- Murphy, P., Davidson, D. R. & Hill, R. E. *Nature* **341**, 156–159 (1989).
- Wilkinson, D. G. et al. *Nature* **341**, 405–409 (1989).
- Hunt, P. et al. *Nature* **353**, 861–864 (1991).
- Sundin, O. H. & Eichele, G. *Genes Dev.* **4**, 1267–1276 (1990).
- Frohmann, M., Boyle, M. & Martin, G. *Development* **110**, 589–608 (1990).
- Maden, M. et al. *Development* **111**, 35–44 (1991).
- Hamburger, V. & Hamilton, H. *J. Morph.* **88**, 49–92 (1951).
- Mangold, O. *Naturwissenschaften* **21**, 761–766 (1933).
- Holtfreter, J. *Wilhelm Roux Arch. Entw. Mech. Org.* **134**, 446–550 (1936).
- Hemmati-Brivanlou, A., Stewart, R. M. & Harland, R. M. *Science* **250**, 800–802 (1990).
- Sharp, C. R. & Gurdon, J. B. *Development* **109**, 765–774 (1990).
- Ruiz i Altaba, A. *Development* **108**, 595–604 (1990).
- Murphy, P. & Hill, R. E. *Development* **111**, 61–74 (1991).
- Lufkin, T. et al. *Cell* **66**, 1105–1119 (1991).
- Chisaka, O., Musci, T. S. & Capocchi, M. R. *Nature* **355**, 516–520 (1992).
- Wilkinson, D. & Green, J. in *Postimplantation Mouse Embryos, a Practical Approach* (eds Rickwood, D. & Cockcroft, D. L.) 155–171 (IRL, Oxford 1990).
- Whiting, J. et al. *Genes Dev.* **5**, 2048–2059 (1991).

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Hormonal stimulation of adenylyl cyclase through G_i-protein $\beta\gamma$ subunits

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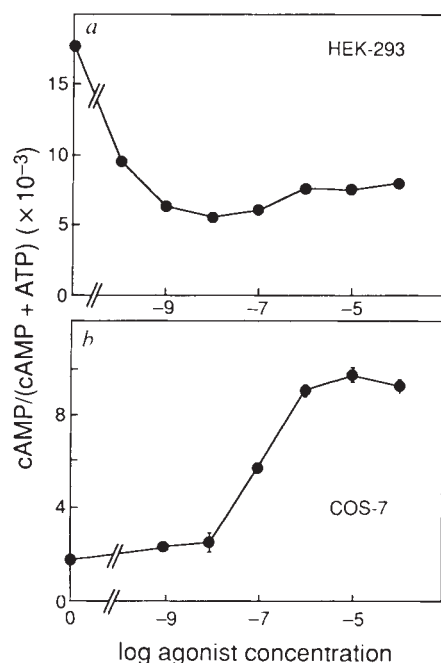
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AGONIST-BOUND receptors activate heterotrimeric ($\alpha\beta\gamma$) G proteins by catalysing replacement by GTP of GDP bound to the α subunit, resulting in dissociation of α -GTP from the $\beta\gamma$ subunits. In most cases, α -GTP carries the signal to effectors, as in hormonal stimulation^{1–4} and inhibition^{5,6} of adenylyl cyclase by α_s and α_i respectively. By contrast, genetic evidence in yeast⁷ and studies in mammalian cells^{8–10} suggest that $\beta\gamma$ subunits of G proteins may also regulate effector pathways. Indeed, of the four recombinant mammalian adenylyl cyclases available for study^{11–14}, two, adenylyl cyclases II and IV, are stimulated by $\beta\gamma$. This effect of $\beta\gamma$ requires costimulation by α_s -GTP^{14,15}. This conditional pattern of effector responsiveness led to the prediction¹⁵ that receptors coupled to many G proteins will mediate elevation of cellular cyclic AMP, provided that G_s is also active. We now confirm this prediction. Coexpression of mutationally active α_s with adenylyl cyclase II converted agonists that act through 'inhibitory' receptors (coupled to G_i) into stimulators of cAMP synthesis. Experiments using pertussis toxin and a putative scavenger of $\beta\gamma$, the α subunit of transducin, suggest that $\beta\gamma$ subunits of the G_i proteins mediated this stimulation. These findings assign a new signalling function to $\beta\gamma$ subunits of G_i proteins, the conditional stimulation of cAMP synthesis by adenylyl cyclase II.

The impetus for the present experiments came from contradictory effects of α_2 adrenoceptors (α_2 -ARs) in different cell types (Fig. 1). The α_2 -AR acts through pertussis toxin (PTX)-sensitive G_i proteins to inhibit adenylyl cyclase in many cells¹⁶. Transient

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expression of α_2 -AR in human embryonic kidney HEK-293 cells, for example, allowed an α_2 -selective agonist to inhibit the cAMP accumulation stimulated by a coexpressed G_s -coupled receptor, the lutropin receptor (LHR) (Fig. 1a; refs 5, 6). By contrast, α_2 -AR stimulation increased cAMP accumulation in both monkey COS-7 cells (Fig. 1b) and PTX-treated Chinese hamster ovary (CHO) cells¹⁷. Although the specific subtypes of adenylyl cyclase expressed in these cell types are not known, a simple explanation is that these results represent responses of different adenylyl cyclases.

The demonstration¹⁵ that $\beta\gamma$ can stimulate adenylyl cyclase II (AC-II) suggested that expression of AC-II in HEK-293 cells could allow the α_2 -AR to stimulate cAMP synthesis through $\beta\gamma$

FIG. 1 Effects of the α_2 -AR agonist, UK-14304, on cAMP accumulation in HEK-293 and COS-7 cells. **a**, Accumulation of [³H]cAMP in HEK-293 cells cotransfected with DNA encoding the lutropin receptor (LHR) and the α_2 -AR. Cells were treated for 30 min with the LHR agonist, human chorionic gonadotropin (hCG, 1 μ g ml⁻¹), in combination with the indicated molar concentrations of UK-14304. **b**, COS-7 cells transfected with the α_2 -AR were treated with the indicated concentrations of UK-14304 for 30 min. UK-14304 (10 μ M) had no effect on cAMP accumulation in either HEK-293 or COS-7 cells that had not been transfected with the α_2 -AR (data not shown). **METHODS.** HEK-293 cells were maintained in minimum essential medium (Earle's) with 10% FCS. COS-7 cells were propagated in Dulbecco's modified Eagles medium (DMEM) with 10% FCS. DNA (LHR-pCIS, ref. 26, 1 μ g; α_2 -AR-pCMV4, ref. 27, 1 μ g) was transfected into HEK-293 cells (10⁶ cells per 60 mm dish) in the presence of DEAE-dextran (400 μ g ml⁻¹) and chloroquine (100 μ M) for 2 h. Cells were shocked with saline (PBS) containing 10% DMSO for 2 min, then washed once with PBS and maintained in growth medium. The COS-7 transfection procedure was identical, except that the DEAE-dextran was 250 μ g ml⁻¹ and transfection was stopped after 4 h. Each 60-mm dish of cells was split into 9 wells of a 24-well plate 24 h after transfection and incubated in medium containing [³H]adenine (1 μ Ci per well). After 18–24 h, cells were washed once with 1 ml assay medium (20 mM HEPES-buffered DMEM without bicarbonate) and then incubated in 0.5 ml assay medium containing 1 mM 1-methyl-3-isobutylxanthine and other drugs, as indicated. Intracellular [³H]cAMP accumulation was estimated by determining the ratio of radiolabelled cAMP to total ATP plus cAMP, as described²⁸. The data represent triplicate determinations in one experiment; three additional experiments gave similar results.

subunits derived from a G protein other than G_s , thereby making HEK-293 behave like COS-7 cells. Indeed, an α_2 agonist did stimulate cAMP accumulation in HEK-293 cells coexpressing AC-II with the α_2 -AR (Fig. 2a). PTX partially blocked this stimulation, suggesting that it was mediated, at least in part, by PTX-sensitive G_i proteins.

To investigate other G_i -coupled inhibitory receptors, we cotransfected HEK-293 cells with DNA encoding the D₂ dopamine receptor (D₂R)¹⁸ and the A₁ adenosine receptor (A₁R). The D₂R, A₁R and α_2 -AR, transiently expressed in HEK-293 cells, mediate agonist inhibition of cAMP accumulation through an endogenous PTX-sensitive G protein, presumably G_i ⁶. In cells cotransfected with AC-II DNA, agonists acting on

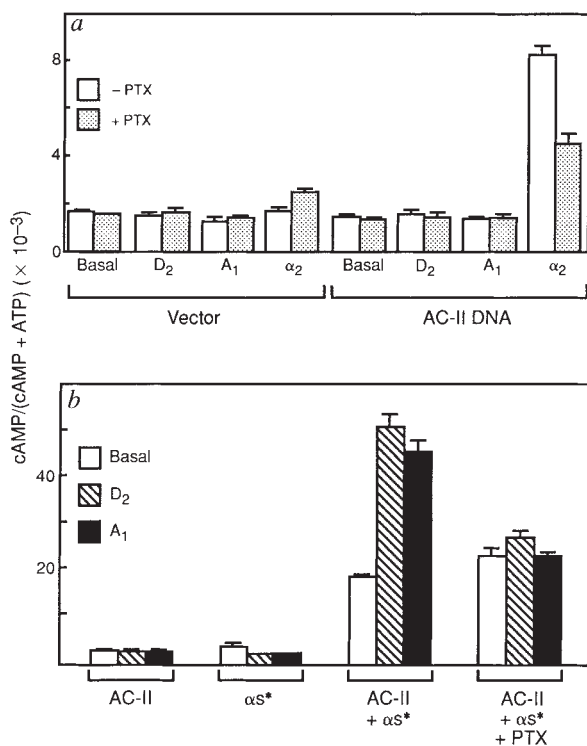
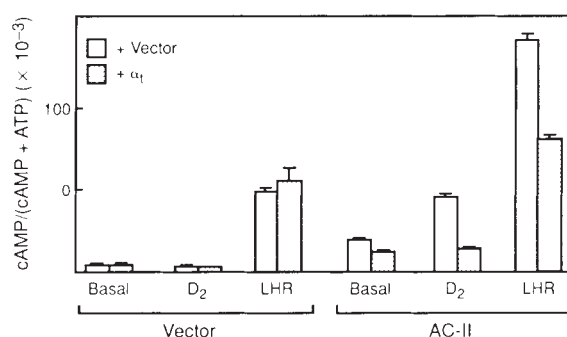


FIG. 2 Accumulation of [³H]cAMP over 30 min in HEK-293 cells treated with: the α_2 -AR agonist UK-14304, 10 μ M (α_2); the D₂R agonist quinpirole, 10 μ M (D₂); or the A₁R agonist (+)-N⁶-(2-phenyl-isopropyl)adenosine, 10 μ M (A₁), as indicated. Cells were treated with or without PTX, 100 ng per well, as indicated, for 18 h before the assay. **a**, All cells were transfected with α_2 -AR-pCIS (1 μ g), A₁R-CDM8 (rat A₁R cDNA, provided by P.D. Garcia, similar to rat A₁R²⁹, 1 μ g), D₂R-pcDNA-I (ref. 8; 1 μ g); other DNAs, including vector (pcDNA-I, Invitrogen, 1 μ g) and AC-II-pcDNA-I (1 μ g), were transfected as indicated. **b**, All cells were transfected with A₁R-CDM8 (1 μ g), D₂R-pcDNA-I (1 μ g), and α_s -Q227L-pcDNA-I (α_s^* , 0.1 μ g); pcDNA-I (1 μ g) and AC-II-pcDNA-I (1 μ g) were transfected as indicated. Methods as Fig. 1 legend. The data represent triplicate determinations in one set of experiments; two additional experiments gave similar results.

FIG. 3 Accumulation of [3 H]cAMP over 30 min in HEK-293 cells treated with the D₂R agonist quinpirole, 10 μ M (D₂), or the LHR agonist hCG, 1 μ g ml⁻¹ (LHR), as indicated. All cells were transfected with LHR-pCIS (1 μ g), D2R-pcDNA-I (1 μ g), and α_s -Q227L-pcDNA-I (α_s^* , 0.1 μ g); AC-II-pcDNA-I (1 μ g) and α_t -pcDNA-I (1 μ g) were transfected as indicated. Vector DNA (pcDNA-I) was added when necessary to bring the total amount of transfected DNA to 4.1 μ g per 60-mm dish. Methods as Fig. 1 legend. The data represent triplicate determinations in a single experiment; two additional experiments gave similar results. Absolute values for cAMP accumulation vary in experiments done on separate days using this protocol (compare hCG responses in Fig. 1 and Fig. 3), but variability within a given experiment is low. Despite variations in maximal cAMP accumulation, experiments done on separate days show similar relative effects for all the agonists and transiently expressed proteins.



the D₂R or the A₁R, unlike the α_2 -AR, failed to stimulate cAMP synthesis (Fig. 2a). Responses of cells expressing recombinant receptors but not AC-II suggested an explanation for the difference between the α_2 -AR and the other two inhibitory receptors. After treatment with PTX, activation of the α_2 -AR but not of the D₂R or the A₁R caused a small rise in cAMP (Fig. 2a). This result suggests that PTX, by blocking the interaction of this receptor with G_i, unmasked a small but productive interaction of the α_2 -AR with G_s. Accordingly, this receptor probably stimulates cAMP synthesis by AC-II by releasing $\beta\gamma$ from G_i and simultaneously interacting with G_s to provide the activated α_s -GTP that is required for stimulation of AC-II activity by $\beta\gamma$ ¹⁵.

This interpretation suggests that $\beta\gamma$ released by activation of other G_i-coupled receptors will also stimulate cAMP synthesis through AC-II if the cells contain active α_s . To provide α_s -GTP without requiring stimulation of G_s by another receptor, we cotransfected cells with a mutationally activated form of α_s (α_s^* , with the Gln 227 $\rightarrow$ Leu (Q227L) mutation¹⁹), which stimulated cAMP synthesis by AC-II (Fig. 2b). As predicted for cells expressing AC-II, agonists acting on either the D₂R or the A₁R further increased this stimulation (Fig. 2b). PTX blocked the stimulatory effects of D₂R and A₁R agonists, indicating that both receptors exerted their effects through a PTX-sensitive G protein, probably G_i.

If this interpretation is correct, it should be possible to counter stimulation of cAMP synthesis by G_i-coupled receptors by expression of a G-protein α subunit that can bind to, and reduce the concentration of, free $\beta\gamma$ derived from G_i. To test this hypothesis, we coexpressed a putative $\beta\gamma$ scavenger, the α_t subunit (α_t)²⁰ of retinal transducin, with the D₂R, α_s^* , and the LHR, in the presence or absence of AC-II. As predicted, α_t completely blocked the stimulatory effect of the D₂R agonist in cells cotransfected with AC-II DNA (Fig. 3). Also, α_t partly inhibited stimulation by the LHR agonist, but only in cells coexpressing AC-II, suggesting that $\beta\gamma$ derived from either G_s or G_i can contribute to stimulation of cAMP synthesis in cells expressing AC-II.

Our results show that expression of AC-II converts agonists that act through three 'inhibitory' G_i-coupled receptors into conditional stimulators of cAMP synthesis, thus extending the findings of Tang and Gilman¹⁵ to include the regulation of cAMP synthesis in intact cells. Other agonists acting on other G_i-coupled receptors, such as somatostatin, serotonin, opioids, acetylcholine and GABA (γ -aminobutyric acid), are likely to promote a similarly conditional activation of AC-II. Indeed, the predominant expression of AC-II in the central nervous system¹³ provides a biochemical explanation for some previously paradoxical observations²¹⁻²⁴. Baclofen and other agonists that act on G_i-coupled receptors augment β -adrenoceptor (β -AR)-stimulated accumulation of cAMP in brain slices, yet by themselves, such agonists do not elevate cAMP. A probable physiological corollary of these changes in cAMP levels (R. Andrade, personal communication) is that isoproterenol, a β -AR agonist,

and cAMP analogues both facilitate synaptic responsiveness in pyramidal cells of the CA1 region of the hippocampus; agents that act through G_i-coupled receptors, including baclofen, substantially augment the facilitating effect of isoproterenol, but have no effect by themselves or in the presence of cAMP analogues.

Our findings exemplify the potential ability of multiple effector isozymes to extend the range and flexibility of transmembrane signalling through G-protein-coupled receptors. The ability of AC-I to respond to stimulation by both G_s and Ca²⁺-calmodulin²⁵ brings cAMP synthesis under the influence of two separate signalling pathways. The sensitivity of AC-II to stimulation by $\beta\gamma$ may have even wider consequences, because it makes cAMP synthesis conditionally susceptible to stimulation by receptors coupled to many G proteins in addition to G_s, potentially including receptors coupled to G_o and to G_q, which mediates stimulation of the phosphoinositide-Ca²⁺ pathway. Moreover, cAMP synthesis is unlikely to be the only effector pathway in which $\beta\gamma$ subunits provide a mechanism for conditional reception of signals from different G proteins. We predict, for example, that phospholipase C isozymes will also prove to be regulated by both α and $\beta\gamma$ subunits of different G proteins. □

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- Birnbaumer, L. A. *Rev. Pharmac. Tox.* **30**, 675-705 (1990).
- Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. & Satoh, T. A. *Rev. Biochem.* **60**, 349-400 (1991).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **348**, 125-132 (1990).
- Freissmuth, M., Casey, P. J. & Gilman, A. G. *FASEB J.* **3**, 2125-2131 (1989).
- Wong, Y. H. et al. *Nature* **351**, 63-65 (1991).
- Wong, Y. H., Conklin, B. R. & Bourne, H. R. *Science* **255**, 339-342 (1992).
- Whiteway, M., Hough, L., Dignard, D., MacKay, V. & Thomas, D. Y. *Cold Spring Harb. Symp. quant. Biol.* **53**, 585-590 (1988).
- Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. *Nature* **325**, 321-326 (1987).
- Jelsema, C. L. & Axelrod, J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3623-3627 (1987).
- Kim, D. et al. *Nature* **337**, 557-560 (1989).
- Krupinski, J. et al. *Science* **244**, 1558-1564 (1989).
- Bakalyar, H. A. & Reed, R. R. *Science* **250**, 1403-1406 (1990).
- Feinstein, P. G. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10173-10177 (1991).
- Gao, B. & Gilman, A. G. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10178-10182 (1991).
- Tang, W.-J. & Gilman, A. G. *Science* **254**, 1500-1503 (1991).
- Cotecchia, S. et al. *J. Biol. Chem.* **265**, 63-69 (1990).
- Fraser, C. M., Arakawa, S., McCombie, W. R. & Venter, J. C. *J. Biol. Chem.* **264**, 11754-11761 (1989).
- Grandy, D. K. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9762-9766 (1989).
- Masters, S. B. et al. *J. Biol. Chem.* **264**, 15467-15474 (1989).
- Medynski, D. C. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4311-4315 (1985).
- Sattin, A., Rall, T. W. & Zanella, J. *J. Pharmac. exp. Ther.* **192**, 22-32 (1975).
- Daly, J. W. et al. *J. Pharmac. exp. Ther.* **212**, 383-389 (1980).
- Karbon, E. W. & Enna, S. J. *Molec. Pharmac.* **27**, 53-59 (1985).
- Hill, D. R. *Br. J. Pharmac.* **84**, 249-257 (1985).
- Tang, W.-J., Krupinski, J. & Gilman, A. G. *J. Biol. Chem.* **266**, 8595-8603 (1991).
- McFarland, K. C. et al. *Science* **245**, 494-499 (1989).
- Guyer, C. A. et al. *J. Biol. Chem.* **265**, 17317-17317 (1990).
- Salomon, Y., Londos, C. & Rodbell, M. *Anal. Biochem.* **58**, 541-548 (1974).
- Mahan, L. C. et al. *Molec. Pharmac.* **40**, 1-7 (1991).

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Close linkage of glucokinase locus on chromosome 7p to early-onset non-insulin-dependent diabetes mellitus

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NON-INSULIN-DEPENDENT diabetes mellitus (NIDDM) is a major health problem, affecting 5% of the world population. Genetic factors are important in NIDDM, but the mechanisms leading to glucose intolerance are unknown^{1,2}. Genetic linkage has been investigated in multigeneration families to localize, and ultimately identify, the gene(s) predisposing to NIDDM. Here we report linkage between the glucokinase locus on chromosome 7p and diabetes in 16 French families with maturity-onset diabetes of the young, a form of NIDDM characterized by monogenic autosomal dominant transmission and early age of onset³. Statistical evidence of genetic heterogeneity was significant, with an estimated 45–95% of the 16 families showing linkage to glucokinase. Because glucokinase is a key enzyme of blood glucose homeostasis⁴, these results are evidence that a gene involved in glucose metabolism could be implicated in the pathogenesis of NIDDM.

Although clinical and metabolic profiles of families with maturity-onset diabetes of the young (MODY) are diverse⁵, most MODY patients present a decreased insulin response to glucose, suggesting a primary pancreatic β -cell defect⁶. Genes whose products seem to be involved in insulin secretion (such as liver or pancreatic β -cell glucose transporter (GLUT2) and glucokinase (GCK), the key enzyme of glucose metabolism in liver and β -cells) are candidate genes for MODY^{7,8}. To determine the genetic regions involved, we tested these candidate genes as well as a candidate region: one gene responsible for MODY has been mapped to the long arm of chromosome 20 in one large American pedigree (lodscore = 5.25; θ = 0.00)⁹ on the basis of its cosegregation with a polymorphic *AluVpA* region in the adenosine deaminase gene (ADA).

We collected genealogical information and clinical data from 16 French MODY families (for pedigrees, see Supplementary Information) ascertained by presence of NIDDM in three consecutive generations (for all but one family in which the older generation was not available), and at least two patients diagnosed before 25 years of age (Table 1, and Figs 1 and 2). Of the 304 individuals examined clinically, 125 were considered affected. Those individuals showed either a plasma glucose concentration higher than 7.8 mmol l⁻¹, 2 h after an oral glucose load (glucose intolerance according to WHO (World Health Organization) criteria), or a fasting plasma glucose higher than 6.1 mmol l⁻¹ in two separate measurements. The latter criteria represent values higher than 3 standard deviations above the mean of the normal population¹¹ and have been used in previous studies¹⁰. Of the 125 affected individuals, 33% presented characteristics of clinical diabetes according

to WHO criteria, namely fasting plasma glucose higher than 7.8 mmol l⁻¹, or treatment with hypoglycaemic agents with previously documented hyperglycaemia at this level. Most diabetic subjects had fasting insulinaemia in the same range as the normoglycaemic relatives but showed a decreased insulin response to an oral glucose load (results not shown).

Genotypes for the GCK, GLUT2 and ADA loci were obtained as described in Tables 1 and 2. For linkage analysis we used LINKAGE programs¹², assuming an autosomal dominant pattern of inheritance under a previously defined segregation model¹³, as described in Table 1. Lodscores were negative in all families characterized for GLUT2, whereas ADA gave positive, but nonsignificant evidence of linkage (lodscore >1) in one family, and negative values in 12 families.

By contrast, the maximum lodscore for the GCK locus was 11.6 over all families for θ = 0.01 (Table 2). One family had a maximum lodscore of 5.9, and three others had lodscores >2. The maximum lodscore for GCK were negative in two families, and a test for linkage heterogeneity with the program HOMOG version 3.1 (ref. 14) was significant (P = 0.0003), suggesting that one or several other loci, not linked to GCK, may be responsible for MODY in some of our families. The maximum likelihood recombination estimate with GCK is 0.00 (0.00–0.05 one-lod-unit support interval) in the linked category of families. The proportion of families in which the disease is linked to GCK was estimated to be 0.8 (0.45–0.95 one-lod-unit support interval). These results were not sensitive to changes in the model assumed for inheritance of the MODY phenotype, as described in Table 1.

The maximum lodscore was found at a recombination fraction of 0.00 in all but one of the 14 families with positive lodscore. Inspection of the data for family F8, which had a maximum lodscore of 2.23 at a recombination fraction of 0.04, revealed a single diabetic individual who did not inherit the allele segregating with the disease in this family. Several arguments suggest that this person presents a different type of diabetes: (1) she is obese (most MODY patients have normal body mass index); (2) she has a more severe disease; (3) a later age of onset of diabetes (52 years); and (4) none of her four children or four grandchildren, all older than 20, is affected. If this individual's affected status is considered as unknown, the maximum lodscore for the family becomes 3.54 at a recombination fraction of zero.

Two large families (F213 and F30) exhibited negative lodscores with GCK. In one of these (F213), patients were found to have an unusual glycaemic profile in which fasting blood glucose was normal, whereas 2-h post-glucose values were consistently in the pathological range. This family also had lower

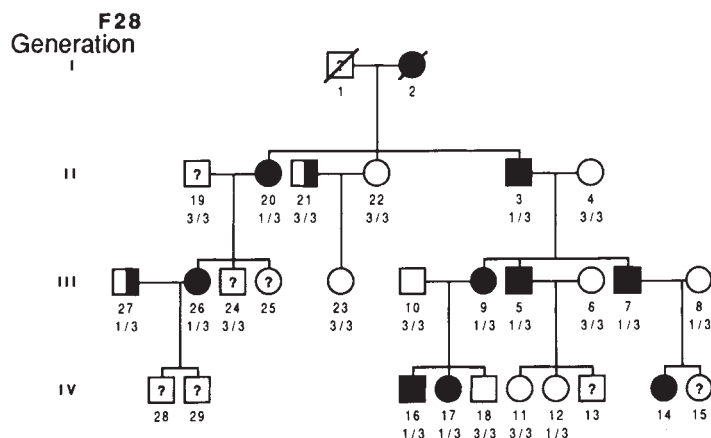


FIG. 1 F28 MODY pedigree. Affected and unaffected individuals are represented by closed and open symbols, respectively, NIDDM by half-closed symbols and untested individuals by question marks. Identification number (first line) and GCK-1 genotype (second line) are shown below each individual.

TABLE 1 Characteristics of the 16 French MODY families

Family number	Total number of subjects	Number of diabetic subjects	ADA lodscore ($\theta=0.00$)	ADA lodscore ($\theta=0.05$)	Marker 66 lodscore ($\theta=0.00$)	Marker 66 lodscore ($\theta=0.05$)	GLUT2 lodscore ($\theta=0.00$)
F8	36	16	-4.86	-1.29	-5.92	-1.85	-4.45
F28	21	8	-2.97	-0.92	-1.80	0.32	-2.23
F30	17	6	1.14	1.24	1.28	1.18	-1.04
F51	103	39	-16.36	-9.89	n.d.	n.d.	-13.62
F85	11	5	-1.89	-0.99	-0.01	-0.01	-1.11
F213	16	4	-2.76	-1.62	-2.84	-1.18	-0.98
F253	7	2	0.97	0.89	-0.02	0.00	-0.53
F313	9	2	-2.69	-0.27	-0.95	-0.81	-1.65
F331	8	3	-2.07	-1.44	-1.98	-0.47	-1.54
F386	6	3	0.54	0.49	0.00	0.00	n.d.
F387	6	4	-0.50	-0.37	-3.66	-1.75	n.d.
F388	7	4	-1.13	-0.45	-1.47	-0.76	n.d.
F391	6	4	-1.11	-0.43	0.00	0.00	n.d.
F392	7	5	-1.05	-0.40	-1.35	-0.65	n.d.
F393	38	17	-10.47	-2.93	-6.11	-0.51	n.d.
F397	6	3	0.55	0.51	0.00	0.00	n.d.

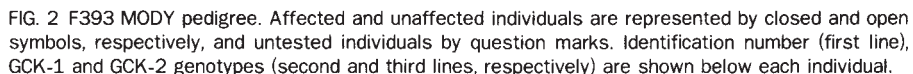
Lodscores for diabetes were obtained versus the ADA *AluVpA/PstI* haplotype and the IP20M66 microsatellite on chromosome 20q, and versus GLUT2 *EcoRI/TaqI/poly(CA)₁₅* microsatellite haplotype. Families were recruited in France in 1990-1991 through a multimedia campaign¹⁹. Families with diabetic members in at least three consecutive generations, autosomal dominant transmission of NIDDM, and several diabetic members diagnosed before 25 years of age, were considered as MODY⁶. The polymorphic *AluVpA* region of the ADA gene was amplified in a polymerase chain reaction (PCR) with a pair of oligonucleotides primers OL3 and OL4 as described²⁰. On amplification, eight alleles were identified with sizes ranging from 180 to 208 base pairs (bp). The ADA intron 2 restriction fragment length polymorphism (*PstI*) was studied by Southern blotting, and was detected by a genomic probe which revealed two alleles of 3.5 kilobases (kb) and 1.6 kb²¹. Marker IP20M66 on chromosome 20q is a (CA)₁₁ microsatellite²², situated at 5 centimorgans (cM) from ADA locus (K. S. Xiang and G. I. Bell, unpublished results). It was detected by PCR (oligomers 66A, 5'-TGCACACCCATGTACACAGACTC-3' and 66M, 5'-GCCAGGTCTCCAACTCTCC-3'). All primers were custom synthesized by GENSET (France). PCR was done by initially denaturing genomic DNA for 5 min at 94 °C, then using 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 68 °C, 30 s extension at 72 °C. Unlabelled PCR products were electrophoresed on 5% denaturing polyacrylamide sequencing gel, and passively blotted on a nylon membrane Hybond N⁺ (Amersham). The filters were then hybridized with one of the two amplification primers [α -³²P]dCTP-labelled by terminal transferase (Boehringer Mannheim). After hybridization (3 h) the filters were washed and autoradiographed for 1-2 h. The results showed an eight-allele polymorphism (three frequent alleles) in the 198-220 bp range. The GLUT2 *EcoRI* and *TaqI* RFLPs were studied by Southern blotting, and were detected by a complementary DNA probe as described^{23,24}. The GLUT2 microsatellite was detected as described²⁵. The lodscores for both sexes were computed together ($\theta_{\text{male}} = \theta_{\text{female}}$). We assumed a population prevalence of the MODY gene of 0.0001, an age-related penetrance of 0.30 for individuals below 10 years old, of 0.65 between 10 and 20 years old, and 0.90 for subjects older than 20 years, and 1% phenocopy frequency. Results were unaffected by changes in the genetic models (modification to full or lower penetrances, changes in gene frequency and phenocopy frequency, subjects with impaired fasting glucose <7.8 mmol l⁻¹ considered as unknown).

TABLE 2 Lodscores for the MODY gene in 16 pedigrees versus the glucokinase microsatellite tandem repeats localized to chromosome 7p (refs 26, 27)

Recombination fraction	0.00	0.01	0.02	0.03	0.04	0.05	0.10	0.20	0.30	0.40
Total lodscore	11.59	11.63	11.60	11.54	11.46	11.33	10.67	8.40	5.58	2.58
F8	2.03	2.14	2.19	2.22	2.23	2.23	2.12	1.65	1.06	0.47
F28	2.18	2.14	2.10	2.06	2.02	1.97	1.75	1.28	0.78	0.31
F30	-2.21	-1.99	-1.82	-1.69	-1.57	-1.47	-1.10	-0.65	-0.35	-0.15
F51	5.89	5.77	5.65	5.53	5.41	5.29	4.69	3.50	2.30	1.09
F85	0.89	0.87	0.85	0.83	0.81	0.78	0.76	0.74	0.72	0.03
F213	-2.68	-2.65	-2.61	-2.54	-2.45	-2.34	-1.74	-0.88	-0.39	-0.11
F253	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.00
F313	0.46	0.44	0.42	0.41	0.39	0.37	0.35	0.34	0.32	0.01
F331	0.28	0.27	0.27	0.27	0.26	0.26	0.23	0.19	0.13	0.07
F386	0.28	0.28	0.27	0.27	0.26	0.26	0.24	0.19	0.14	0.07
F387	0.31	0.30	0.30	0.30	0.30	0.30	0.29	0.25	0.19	0.11
F388	0.32	0.31	0.30	0.29	0.28	0.27	0.26	0.25	0.24	0.00
F391	0.55	0.53	0.52	0.51	0.50	0.48	0.42	0.28	0.15	0.04
F392	0.42	0.40	0.39	0.37	0.36	0.34	0.26	0.13	0.05	0.01
F393	2.27	2.22	2.18	2.13	2.09	2.04	2.00	1.95	1.90	0.00
F397	0.55	0.55	0.54	0.53	0.52	0.51	0.47	0.37	0.27	0.14

The polymorphic glucokinase (CA) repeat (GCK1) was detected by PCR with primers 5'-CCCACACCAAACTGCCTGTATTAG-3' and 5'-TTGGTCAGTGTAGGCT-GAACTCATG-3'²⁶. PCR was done by initially denaturing genomic DNA for 6 min at 92 °C, followed by 1 min annealing at 68 °C, 30 s extension at 72 °C, then using 30 cycles with 40 s denaturation at 92 °C, 30 s annealing at 68 °C, 30 s extension at 72 °C, followed by a 7 min final extension at 72 °C. Three alleles ranging in length from 180 to 205 bp were observed in our pedigrees, and the observed heterozygosity is 42%. Families uninformative for GCK1 were genotyped for a second microsatellite of the glucokinase locus (GCK2; Y.T., K. Chen and M.A.P., unpublished results) showing four alleles in our pedigrees. GCK2 was detected by PCR, with primers 5'-CTGTGCCATGGTTATATAAGAGAAG-3' and 5'-AAACAGATACGCTTCATCCTGATTC-3'. PCR reaction was done under the same conditions as for the marker IP20M66. PCR products were analysed and linkage analysis and lodscores were calculated as described in Table 1.

In view of the difficulties of assaying the *in vivo* activity of glucokinase in humans, it is important to find more informative markers for this region so that the remaining uninformative families can be characterized with respect to this locus. But for a definitive demonstration that this gene product is involved in this pathology, the predisposing mutations(s) must be identified. □



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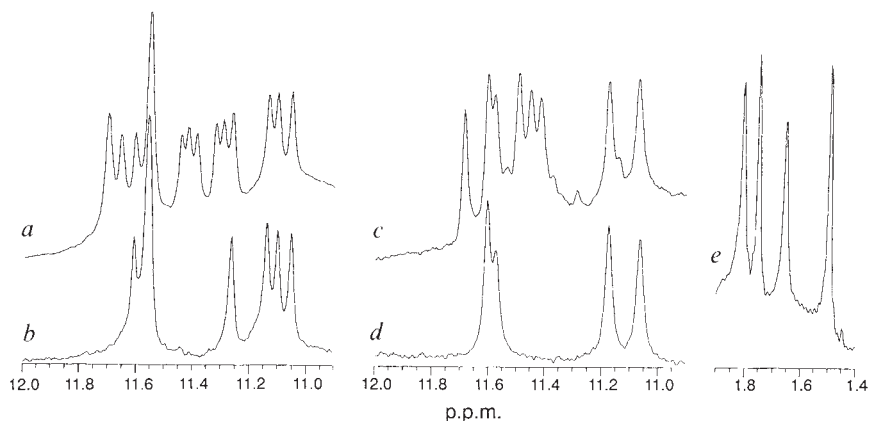
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1. Rahilly, S. O., Wainscoat, J. S. & Turner, R. C. *Diabetologia* **31**, 407-414 (1988).
2. Rich, S. S. *Diabetes* **39**, 1315-1319 (1990).
3. Tattersall, R. B. & Fajans, S. S. *Diabetes* **24**, 44-53 (1975).
4. Magnuson, M. A. *Diabetes* **39**, 523-527 (1990).
5. Fajans, S. S. *Diab. Metab. Rev.* **5**, 579-606 (1989).
6. Fajans, S. S. *Diabetes Care* **13**, 49-64 (1990).
7. Unger, R. H. *Science* **251**, 1200-1205 (1991).
8. Chanson, Ph., Ferre, P. & Timsit, J. *Méd. Sci.* **7**, 336-345 (1991).
9. Bell, G. I. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 1484-1488 (1991).
10. O'Rahilly, S. *et al. Diabetologia* **31**, 792-797 (1988).
11. Multicentre Study (coord. Turner, R. C.) *Diabetologia* **24**, 404-411 (1983).
12. Lathrop, G. M., Lalouel, J. M., Julier, C. & Ott, J. *Am. J. hum. Genet.* **37**, 482-498 (1985).
13. Rahilly, S. O. & Turner, R. C. *Diab. Med.* **5**, 224-229 (1988).
14. Ott, J. in *Human Genetic Diseases: A Practical Approach* (ed. Davies, K. E.) 19-32 (IRL, Oxford, 1991).
15. De Fronzo, R. A. *Diabetes* **37**, 667-687 (1988).
16. Meglasson, M. A. & Matschinsky, F. M. *Am. J. Physiol.* **246**, 1-13 (1984).
17. Matschinsky, F. M. *Diabetes* **39**, 647-652 (1990).
18. Magnuson, M. A. & Shelton, K. D. *J. biol. Chem.* **27**, 15936-15942 (1989).
19. Froguel, Ph., Velho, G. & Passa, Ph. *Diabetologia* **34**, 685 (1991).
20. Economou, E. P., Bergen, A. W., Warren, A. C. & Antonarakis, S. E. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2951-2954 (1990).
21. Tzall, S., Ellenbogen, A., Eng, F. & Hirschhorn, R. *Am. J. hum. Genet.* **44**, 864-875 (1989).
22. Hazan, J., Dubay, C., Pankowiak, M. P., Becuue, N. & Weissenbach, J. *Genomics* (in the press).
23. Matsutani, A., Koranyi, L., Cox, N. & Permutt, A. *Diabetes* **39**, 534-542 (1990).
24. Froguel, Ph., Vionnet, N., Lesage, S., Velho, G. & Cohen, D. *Nucleic Acids Res.* **19**, 5799 (1991).

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FIG. 1 One-dimensional 500 MHz ^1H NMR spectra of the imino proton resonances of: Oxy-3.5 at 20 °C in 90% $\text{H}_2\text{O}/10\%$ D_2O (a) and after 26 days in D_2O (b); and Oxy-1.5 at 10 °C in 90% $\text{H}_2\text{O}/10\%$ D_2O (c) and after 14 days in D_2O (d); methyl resonances of Oxy-1.5 in 90% $\text{H}_2\text{O}/10\%$ D_2O (e). Samples are 2 mM strand (Oxy-3.5) and 4 mM strand (2 mM dimer) (Oxy-1.5), pH 7, in 50 mM NaCl (400 μl). DNA was synthesized on an ABI 381 synthesizer using β -cyanoethyl phosphoramidite chemistry and purified as described^{13,14}. Lyophilized DNA was dissolved in 50 mM NaCl and the pH adjusted with NaOH. Samples were transferred to a 5-mm NMR tube, lyophilized or dried under a stream of N_2 , and redissolved in 90% $\text{H}_2\text{O}/10\%$ D_2O . Small amounts of alternative conformations were reduced by heating to $\sim 90^\circ\text{C}$ followed by slow cooling. Samples were transferred to D_2O by drying as before and redissolving in 99.996% D_2O . Spectra of Oxy-3.5 and Oxy-1.5 in 90% H_2O were acquired with a 11° spin-echo pulse sequence¹⁵ and spectra in D_2O were acquired with presaturation of the residual HDO. The acquisition parameters were



8,000 Hz spectral width, 2-s recycle delay, 256 acquisitions. Spectra were processed with a gaussian line broadening of 3 Hz.

This arrangement differs from earlier models in which the strands are alternately all *syn* or all *anti*^{2,3,5}. The T_4 loops in Oxy-1.5 are on opposite ends of the quadruplex and loop diagonally across the G-quartet, resulting in adjacent strands being alternately parallel and antiparallel.

T_4G_4 repeats of $3\frac{1}{2}$ and $1\frac{1}{2}$ were chosen so that the folded structures, once formed, would not have a dangling 5' T_4 end. A unimolecular folded structure with two central G-quartets was proposed² for a related oligonucleotide (Oxy-4) on the basis of gel electrophoresis, dimethyl sulphate methylation and ultraviolet crosslinking. One-dimensional ^1H NMR spectra of the imino-proton resonances of Oxy-3.5 are shown in Fig. 1a. Sixteen hydrogen-bonded imino resonances are seen in the Oxy-3.5 spectrum, consistent with the formation of a single folded structure with four G-quartets (Fig. 3b). Although the observed number of resonances would also be consistent with a symmetrical four-stranded quadruplex or a quadruplex composed

of a symmetrical dimer, the linewidths of the non-exchangeable resonances (<9 Hz) are indicative of a unimolecular folded structure. Only eight hydrogen-bonded imino resonances (Fig. 1c) and four methyl resonances (Fig. 1e) are observed for Oxy-1.5. For both molecules, the G iminos as well as some of the amino protons show unusually slow exchange with water, with half of the iminos remaining after weeks in D_2O (Fig. 1b, d).

The relatively simple appearance of the Oxy-1.5 spectrum indicates that only one hydrogen-bonded conformation predominates. This structure cannot be a simple hairpin, because there is no base-pairing scheme that would give rise to eight hydrogen-bonded imino protons along with hydrogen-bonded amino protons. In nuclear Overhauser effect spectroscopy (NOESY) of Oxy-1.5 in H_2O (not shown), six of the expected eight amino pairs appear as well resolved pairs of resonances. This is unusual for G aminos in DNA, which usually appear as

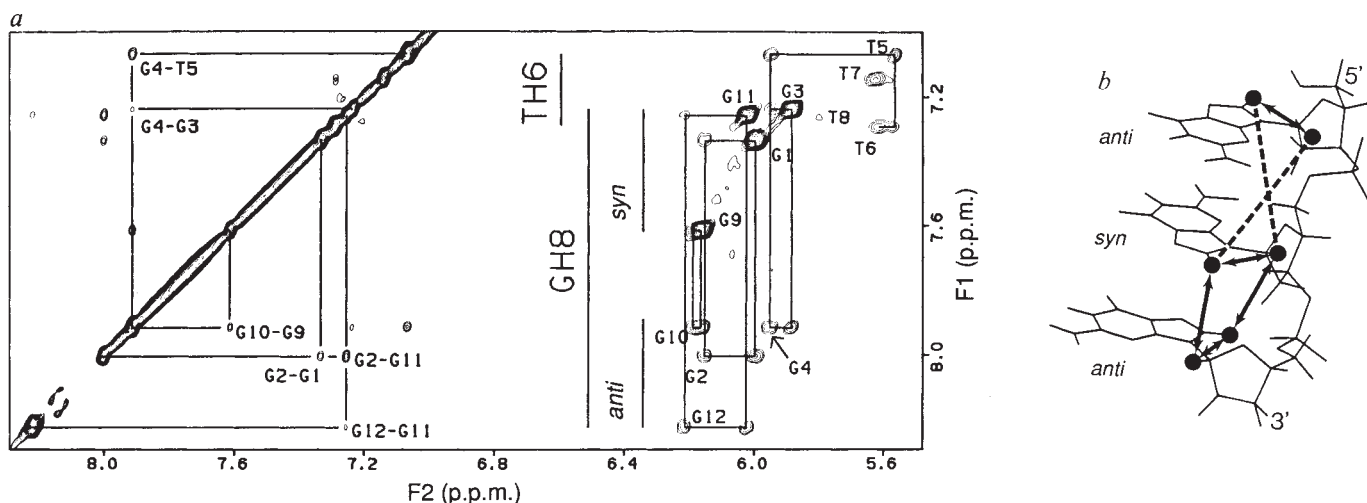


FIG. 2 a, Portion of NOESY spectrum containing the base-base and base- $\text{H}1'$ crosspeaks of Oxy-1.5 at 20 °C in D_2O . Sample is the same as Fig. 1 except the pH was adjusted to 6. Observed base-base and base- $\text{H}1'$ connectivities are illustrated with solid lines. Each 5'-*syn-anti*-3' nucleotide pair shows base- $\text{H}1'$ and base-base crosspeaks. Assignments of the exchangeable and non-exchangeable resonances of Oxy-1.5 were determined from NOESY¹⁶, P-COSY¹⁷ and HOHAHA¹⁸ spectra and will be discussed elsewhere. Because no base- $\text{H}1'$ sequential connectivities are observed between *anti-syn* steps, standard sequential assignment¹⁹ of these molecules was not possible. Assignments of T6-8 are tentative. b, Schematic of alternating *anti* and *syn* G nucleotides along a strand. The H8- $\text{H}1'$ interproton connectivities which would give rise to NOEs (<5 Å) are illustrated with dark

lines. The shortest distance is the intranucleotide GH8- $\text{H}1'$ on the *syn* base (~ 2.4 Å). All other short distances are around 4 Å. H8- $\text{H}1'$ distances which are too long to give rise to NOEs are illustrated with dashed lines. These distances are 7-9 Å. We were unable to construct any left-handed structures that would satisfy the NOE constraints. The NOESY spectrum was acquired in the hypercomplex phase-sensitive mode²⁰ with the standard pulse sequence¹⁶, with a spectral width of 4,000 Hz, 275 t_1 values, 32 scans per acquisition, 2,000 complex points in t_2 , and a mixing time of 200 ms. The spectrum was zero-filled to 2,000 complex points in t_1 and was apodized with a skewed sine bell (60, 1.1) in both dimensions using FELIX (Hare Research, Inc.).

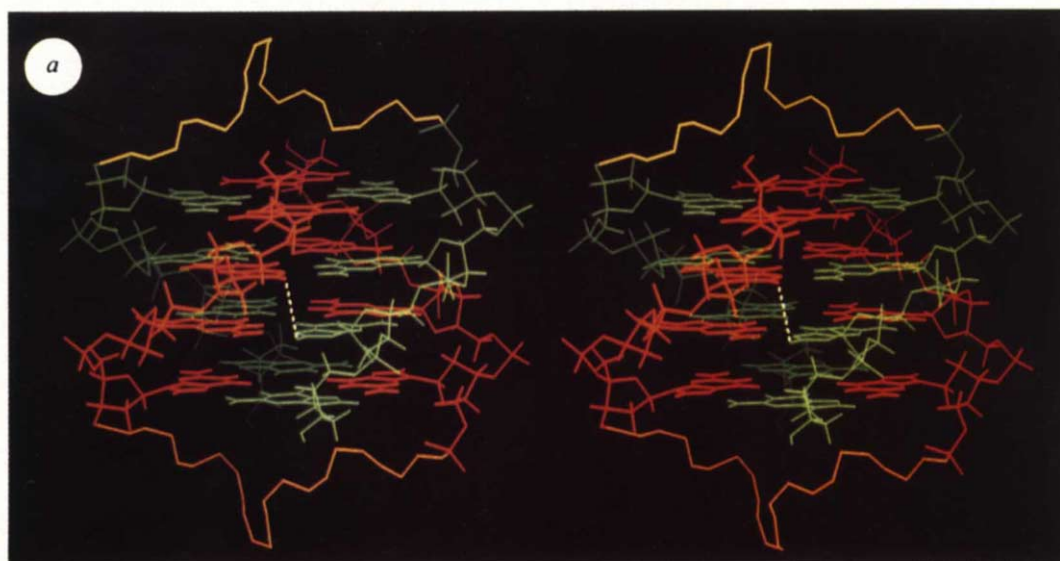
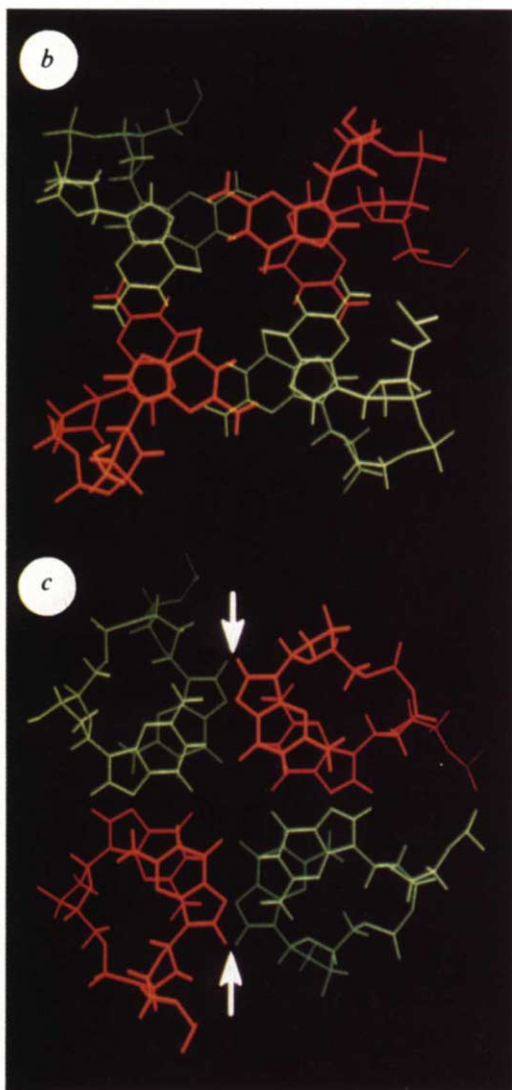


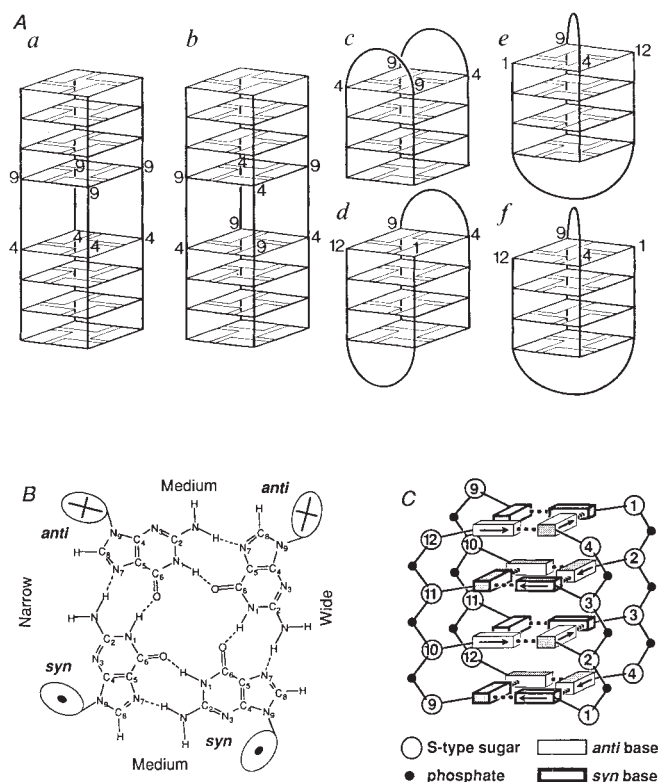
FIG. 4 Molecular model for the structure of Oxy-1.5 showing *a*, stereo view of the medium groove. One of the two (symmetrical) intermolecular G2H8-G11H8 NOE connectivities is illustrated by the dotted line. The structure has a dinucleotide repeating unit. *b*, Stacking of outer two G-quartets. *c*, Stacking of central two G-quartets. Arrows point to the G2 and G11 H8s. Note that there is little twist between 5'*syn* and 3'*anti* guanines, but a large twist between 5'*anti* and 3'*syn* guanines. The model was initially built using INSIGHT II (Biosym Technologies Inc.) and FRODO and refined using XPLOR²⁴ dynamics and rigid body minimization. Dihedral bond angle constraints were used to restrain the guanosine sugar conformations. The relaxation matrix option of XPLOR²⁵ was used for all resolvable non-loop NOE volumes. Some NOE constraints were used in the initial construction of the loops, but no experimental data for the loops was used during the refinement.



a single broad resonance owing to fast rotation around the C-N bond⁹. For each of these aminos, crosspeaks are observed to an imino resonance and from the hydrogen-bonded (lower field) amino to a GH8 resonance, confirming the G-quartet hydrogen bonding scheme. Similar crosspeak patterns are observed for Oxy-3.5 (spectrum not shown). The more slowly exchanging imino and amino resonances in Oxy-1.5 have been assigned to the inner two G-quartets in the structure. Formation of a structure containing G-quartets which gives rise to only eight imino and four methyl resonances is consistent only with a symmetrical structure.

The symmetrical structure of the Oxy-1.5 quadruplex greatly simplified assignments by halving the number of observed resonances, and we therefore focused on this structure. A portion of a NOESY spectrum of Oxy-1.5 showing the region containing the base-base and base-H1' crosspeaks is shown in Fig. 2*a*. Both in Oxy-1.5 and in Oxy-3.5 (not shown), one half of the G nucleotides show intense H8-H1' NOE crosspeaks, indicating that they are in the *syn* conformation^{10,11}. Each of these nucleotides shows a single sequential connectivity to a G nucleotide in the *anti* conformation. These connectivities are also traced in the base-H2', H2'' and other regions (not shown). Thus, pairs of sequential 5'-G*syn*-G*anti*-3' nucleotides are identified. These crosspeak patterns establish unambiguously that the bases are alternately *syn* and *anti* along each strand. There are no sequential base-H1', H2', H2'' connectivities from 5'-G*anti*-G*syn*-3'. The relative interproton distances for 5'-*syn*-*anti*-3' steps and 5'-*anti*-*syn*-3' steps are illustrated schematically in Fig. 2*b*. The crosspeak patterns observed in NOESY spectra in D₂O for Oxy-3.5 (not shown) are similar to those for Oxy-1.5, indicating that the two molecules form related structures.

FIG. 3 A, Schematic illustrating possible symmetrical structures which Oxy-1.5 could form which contain G-quartets and alternating *syn-anti* bases along each strand: a, b, structures formed from four Oxy-1.5 strands. The strands can be (a) all parallel or (b) alternate strands can be antiparallel; (c), dimer of Oxy-1.5 with T loops both at the same end; (d), dimer of Oxy-1.5 with T loops at opposite ends connecting adjacent strands, (e, f) dimer of Oxy-1.5 with loops diagonally across G-quartet. Note that structures e and f are not composed of two hydrogen-bonded hairpins; e and f differ by the polarity of the second strand. Two additional structures not shown are the same as c and d except the polarity of both strands is reversed. Other possible G-quartet DNA structures, such as any of a–d with the polarity of one strand reversed, would not be symmetrical. A tetramolecular complex of $G_2T_5G_2$ with alternating G_{syn} and G_{anti} nucleotides was recently reported²⁶. B, Illustration of the base pairing in the G-quartet, glycosidic torsion angles, location of grooves, and strand directions (+, 5' and –, 3' ends). Note that although the individual G-quartet is asymmetrical the structure of Oxy-1.5 as a whole is symmetrical. C, Summary of the experimentally determined Oxy-1.5 G-quadruplex structure. A medium groove view is drawn. There is a 2-fold axis of symmetry which is perpendicular to the helix axis and passes through the narrow and wide grooves. All of the GH8 protons in the narrow and wide grooves are from *anti* and *syn* bases, respectively. Those in the medium grooves alternate between *syn* and *anti*. The alternation of G-quartets between two orientations, that is head-to-head stacking of pairs of tetrads^{21,22} is illustrated by the shading on the schematic G-bases. The arrows illustrate the direction of the proton donors and acceptors. The pattern of hydrogen-bond donors and acceptors alternates from one G-quartet to the other, between donor–acceptor and acceptor–donor. This differs from G-quadruplex model structures which are alternately all *syn* or all *anti* along each strand (all donor–acceptor). The thymine nucleotides in the loop, not illustrated, are all *anti*. Conformations of the sugars were determined by analysis of P.COSY¹⁷ spectra (not shown) using the simulation package CHEOPS²⁷ to determine coupling constants and PSEUROT²³ to determine dihedral bond angles.



Theoretically possible symmetrical (non-polymeric) right-handed structures for Oxy-1.5 that contain G-quartets are illustrated schematically in Fig. 3A. In the NOESY spectrum of Oxy-1.5 in D_2O , NOE crosspeaks are observed between four different (non-symmetrical) guanines (two *syn* and two *anti*) and resonances from three of the thymines (Fig. 2a and regions not shown). The parallel and antiparallel four-stranded structures in Fig. 3A(a,b) would each have only two different (non-symmetrical) guanines (bases 4 and 9) next to the thymines and thus cannot be correct. In addition, the linewidths for Oxy-1.5 are slightly narrower than for Oxy-3.5, which would also be inconsistent with the structures of higher molecular mass. A symmetrical bimolecular quadruplex with both loops on the same side (Fig. 3A(c)) also has only two different guanines next to thymines. Only the symmetrical bimolecular quadruplexes with loops at opposite ends (Fig. 3A(d-f)) are consistent with this data for Oxy-1.5. A *syn* GH8 to *anti* GH8 NOE crosspeak that does not correspond to a sequential 5'-*syn-anti*-3' connectivity, and thus must arise from an interstrand interaction, is observed in all spectra of Oxy-1.5 (Fig. 2a), as well as Oxy-3.5. This has been identified in Oxy-1.5 as an NOE between *anti*G2H8 on one oligonucleotide and *syn*G11H8 on the other (symmetry related) oligonucleotide (Fig. 4). This type of inter-strand connectivity (that is, *anti* to *syn*) is only consistent with parallel stranded and thus diagonally looped structures. The specific guanine residues involved indicate that the structure in Fig. 3A(f) is correct. Presumably, the preference for one diagonally looped isomer over the other is an effect of the folding process. The population of minor conformations present in the samples may be one or more of the alternative structures illustrated or other non-symmetrical structures.

The structural features determined from the NMR data for the G-quartet core of Oxy-1.5 are summarized schematically in Fig. 3B, C. The guanines alternate *syn* and *anti* along each strand, beginning with a 5'*Gsyn*. A consequence of the alternation of *syn* and *anti* nucleotides is that the G-quartets alternate between two orientations (Fig. 3C) and the pattern of hydrogen-bond donors and acceptors also alternates from one G-quartet

to the next. All of the deoxyribose rings have S-type (near C2'-endo) conformations. There are several unexpected features of the quadruplex structure which are a consequence of the looping of the thymines diagonally across the quartet. Each guanine is hydrogen bonded only to guanines from the other oligonucleotide. Adjacent strands are alternately parallel and antiparallel with the result that glycosidic torsion angles around each G-quartet are *syn-syn-anti-anti*. Results on Oxy-3.5 indicate that it forms an analogous structure in which only the central four Ts loop diagonally across the quartet at one end of the helix and the other Ts form two loops along opposite edges of the quartet at the other end.

A molecular model for the Oxy-1.5 structure that incorporates the information discussed, as well as some constraints on the loops derived from observed NOEs, is shown in Fig. 4. Other important features of the diagonally looped structure are the grooves, one narrow, two medium and one wide, with the width being determined by the relative orientations of the sugars (Fig. 3B). The two symmetric medium grooves are formed between the two pairs of parallel strands. The narrow and wide grooves are formed between anti-parallel strands.

An important difference between the diagonally looped and edge-looped models for the Oxy-1.5 quadruplex is that the diagonally looped structures are not simple dimers of stem-loop hairpins. Thus, formation of the quadruplex must occur by a pathway other than the dimerization of two Hoogsteen base-paired hairpins. The diagonal looping may also contribute to the stability of the quadruplex because all 32 hydrogen bonds would need to be broken to separate the two molecules. We speculate that this may be a factor in association of telomeres in *Oxytricha*¹². □

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1. Zakian, V. A. *Rev. Genet.* **23**, 579–604 (1989).
2. Williamson, J. R., Raghuraman, M. K. & Cech, T. R. *Cell* **59**, 871–880 (1989).
3. Panyutin, I. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 867–870 (1990).
4. Hardin, C. C., Henderson, E., Watson, T. & Prosser, J. K. *Biochemistry* **30**, 4460–4472 (1991).
5. Sundquist, W. I. & Klug, A. *Nature* **342**, 825–829 (1989).

6. Sen, D. & Gilbert, W. *Nature* **334**, 364–366 (1988).
7. Jin, R., Breslauer, K. J., Jones, R. A. & Gaffney, B. L. *Science* **250**, 543–546 (1990).
8. Guschlbauer, W., Chantot, J.-F. & Thiele, D. *J. biomolec. Struct. Dyn.* **8**, 491–511 (1990).
9. Boelens, R., Scheek, R. M., Dijkstra, K. & Kaptein, R. *J. magn. Reson.* **62**, 378–386 (1985).
10. Feigon, J., Wang, A. H.-J., van der Marel, G. A., van Boom, J. H. & Rich, A. *Nucleic Acids Res.* **12**, 1243–1263 (1984).
11. Patel, D. J., Kozlowski, S. A., Nordheim, A. & Rich, A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1413–1417 (1982).
12. Oka, Y. & Thomas, C. A. Jr *Nucleic Acids Res.* **15**, 8877–8898 (1987).
13. Kintanar, A., Klevit, R. E. & Reid, B. R. *Nucleic Acids Res.* **15**, 5845–5862 (1987).
14. Feigon, J. et al. in *DNA Structures* (eds Lilley, D. M. J. & Dahlberg, J. E.) (Academic, Orlando, in the press).
15. Sklenář, V. & Bax, A. *J. magn. Reson.* **74**, 469–479 (1987).
16. Kumar, A., Ernst, R. R. & Wüthrich, K. *Biochem. biophys. Res. Commun.* **95**, 1–6 (1980).
17. Marion, D. & Bax, A. *J. magn. Reson.* **80**, 528–533 (1988).
18. Davis, D. G. & Bax, A. *J. Am. chem. Soc.* **107**, 2820–2821 (1985).
19. Wüthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
20. States, D. J., Haberkorn, R. A. & Ruben, D. J. *J. magn. Res.* **48**, 286–292 (1982).
21. Borzo, M. & Laszlo, P. *C. hebd. Séanc. Acad. Sci., Paris* **287**, 475–478 (1978).
22. Pinnavaia, T. J. et al., *J. Am. chem. Soc.* **100**, 3625–3627 (1978).
23. de Leeuw, F. A. M. & Altona, C. *J. comput. Chem.* **4**, 428–437 (1983).
24. Brünger, A. T. *X-PLOR (Version 2.1) Manual* 1–291 (Yale Univ., New Haven, CT, 1990).
25. Nilges, M., Habazetti, J., Brünger, A. T. & Holak, T. A. *J. molec. Biol.* **219**, 499–510 (1991).
26. Wang, Y., Jin, R., Gaffney, B., Jones, R. A. & Breslauer, K. J. *Nucl. Acids Res.* **19**, 4619–4622 (1991).
27. Macaya, R. F., Schultze, P. & Feigon, J. *J. Am. chem. Soc.* **114**, 781–783 (1992).

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Long-range correlations in nucleotide sequences

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DNA SEQUENCES have been analysed using models, such as an n -step Markov chain, that incorporate the possibility of short-range nucleotide correlations¹. We propose here a method for studying the stochastic properties of nucleotide sequences by constructing a 1:1 map of the nucleotide sequence onto a walk, which we term a 'DNA walk'. We then use the mapping to provide a quantitative measure of the correlation between nucleotides over long distances along the DNA chain. Thus we uncover in the nucleotide sequence a remarkably long-range power law correlation that implies a new scale-invariant property of DNA. We find such long-range correlations in intron-containing genes and in nontranscribed regulatory DNA sequences, but not in complementary DNA sequences or intron-less genes.

For the conventional one-dimensional random walk model, a walker moves either up ($u(i) = +1$) or down ($u(i) = -1$) one unit length (u) for each step i of the walk². For the case of an uncorrelated walk, the direction of each step is independent of the previous steps. For the case of a correlated random walk, the direction of each step depends on the history ('memory') of the walker. The DNA walk is defined by the rule that the walker steps up ($u(i) = +1$) if a pyrimidine occurs at a position a linear distance i along the DNA chain, whereas the walker steps down ($u(i) = -1$) if a purine occurs at position i . Does such a walk display only short-range correlations (as in an n -step Markov chain) or long-range correlations (as in critical phenomena and other scale-free 'fractal' phenomena).

This DNA walk provides a graphical representation for each gene and permits the degree of correlation in the nucleotide sequence to be directly visualized, as in Fig. 1. Figure 1 naturally motivates a quantification of this correlation by calculating the

'net displacement' (y) of the walker after l steps, which is the sum of the unit steps $u(i)$ for each step i ,

$$y(l) = \sum_{i=1}^l u(i). \quad (1)$$

An important statistical quantity characterizing any walk² is the root mean square fluctuation $F(l)$ about the average of the displacement; $F(l)$ is defined in terms of the difference between the average of the square and the square of the average,

$$F^2(l) = \overline{[\Delta y(l) - \overline{\Delta y(l)}]^2} \\ = \overline{[\Delta y(l)]^2} - [\overline{\Delta y(l)}]^2, \quad (2a)$$

of a quantity $\Delta y(l)$ defined by

$$\Delta y(l) = y(l_0 + l) - y(l_0). \quad (2b)$$

Here the bars indicate an average over all positions l_0 in the gene. Operationally, this is equivalent to (1) taking a set of calipers set for a fixed distance l , (2) moving the beginning point sequentially from $l_0 = 1$ to $l_0 = 2$ and so on (3) calculating the quantity $\Delta y(l)$ (and its square) for each value of l_0 , and (4) averaging all of the calculated quantities to obtain equation (2a).

The mean-square fluctuation is related to the auto-correlation function $C(l) = \overline{u(l_0)u(l_0 + l)} - [\overline{u(l_0)}]^2$ through the relation³ $F^2(l) = \sum_{i=1}^l \sum_{j=1}^l C(j-i)$. Because F is related to C by a summation, the fluctuation in F is substantially reduced compared with the fluctuation in C . Hence measurement of F leads to a more reliable characterization of the DNA walk than measurement of C .

The calculation of $F(l)$ can distinguish three possible types of behaviour. (1) If the nucleotide sequence were random, then $C(l)$ would be zero on average (except $C(0) = 1$), so $F(l) \sim l^{1/2}$ (as expected for a normal random walk). (2) If there were a local correlation extending up to a characteristic range R (such as in Markov chains), then $C(l) \sim \exp(-l/R)$; nonetheless the asymptotic behaviour $F(l) \sim l^{1/2}$ would be unchanged from the purely random case³. (3) If there is no characteristic length (that is, if the correlation were 'infinite-range'), then the scaling property of $C(l)$ would not be exponential, but would instead be a power law function, and the fluctuations will also be described by a power law

$$F(l) \sim l^\alpha \quad (3)$$

with $\alpha \neq \frac{1}{2}$.

Figure 1a shows a typical example of an intron-containing gene. The DNA walk has an obviously very jagged contour which corresponds to long-range correlations. Our calculation of $F(l)$ for this gene is shown in Fig. 2a. That the data are linear over three decades on this double logarithmic plot confirms that $F(l) \sim l^\alpha$. We calculated a least-squares fit and find a straight line with slope $\alpha = 0.67 \pm 0.01$.

Figure 1b shows the DNA walk for the cDNA sequence of this same gene, whereas Fig. 1c shows the data for a typical intron-less gene. In contrast to Fig. 1a, these intron-free sequences have less jagged contours, suggesting a shorter range correlation. In almost all the intron-less sequences studied, purine-rich regions (compared with the average concentration over the entire strand) alternate with pyrimidine-rich regions, corresponding to the 'up-hill' and 'down-hill' portions of the DNA walk. To take into account the fact that the concentrations of purines and pyrimidines are not constant throughout the single-strand nucleotide sequence, we partitioned each DNA walk representation into segments demarcated by the global maximum ('max') and minimum ('min') displacements and analysed the fluctuation in each segment. Figure 2a also shows the data for the cDNA, and a least-squares fit gives a straight line with slope $\alpha = 0.49 \pm 0.01$. To ensure we did not introduce a bias by this 'min-max partitioning', we also used the same partitioning procedure to analyse the full gene and find no change in the exponent α .

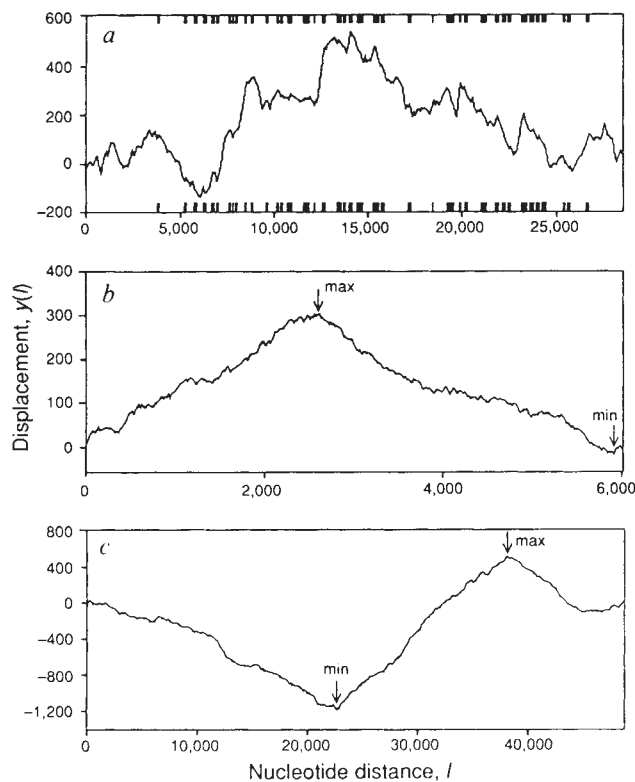


FIG. 1 The DNA walk representations of intron-rich human β -cardiac myosin heavy-chain gene sequence (a) its cDNA (b), and the intron-less bacteriophage λ DNA sequence (c). Note the more complex fluctuations for the intron-containing gene in a compared with the intron-less sequences in b and c. Heavy bars denote coding regions of the gene. So that the graphical representation was not affected by the global differences in concentration between purines and pyrimidines, DNA walk representations were plotted so that the end point has the same vertical displacement as the starting point (for the statistical analysis, we use the original definitions (1)–(3), without any adjustment of vertical displacement). The minimum (min) and maximum (max) points on the landscape are denoted by arrows, and their application in the analysis is described in the text. For almost all intron-less genes and cDNA sequences studied, there appear regions with one strand bias, followed by regions of a different strand bias. The fluctuation on either side of the overall strand bias is random, a fact that is plausible by visual inspection of the DNA walk representations.

where we average the actual fluctuations over the two sets of entries in Table 1. Thus, the calculation of $F(l)$ for the DNA walk representation provides a new, quantitative method to distinguish genes with multiple introns from intron-less genes and cDNAs based solely on their statistical properties.

To determine if the long-range correlations we observe could be due to long repetitive nucleotide sequences, we tested whether the correlations would persist when the nucleotides blocks of size L were shuffled. Specifically, we divided the nucleotide sequence into many small segments (each of length $L = 100$) and then randomized the purine–pyrimidine order in each segment. We find, as expected, that the long-range correlation does persist. This calculation shows that the long-range correlation is a 'global' property of the nucleotide organization and is independent of the details of any repeating sequences. The calculation is equivalent to a 'renormalization' of the sequence in which each individual segment is replaced by the corresponding uniform concentration of nucleotides.

The four nucleotides (adenine, guanine, thymine and cytosine) were analysed using the DNA walk and we observed long-range correlations in intron-containing genes. We find the most robust fits to power law scaling by computing the DNA walk for nucleotide class (purine versus pyrimidine) rather than for any specific nucleotide.

To test if our results could be artefacts of a strand bias (that is, that over some distance one strand is more purine-rich), we studied many 'artificial' nucleotide sequences with and without strand bias. We find the same exponent α for both cases; the effect of a strand bias is to give a non-zero value for the quantity $\Delta y(l)$, but this quantity is subtracted in the actual calculations of the mean deviation from the average in equation (2a). We

To see if this scaling behaviour is universal, we applied our analysis to a large number of representative genomic and cDNA sequences across the phylogenetic spectrum, some of which are shown in Table 1. The myosin heavy-chain family is particularly useful because it encompasses a number of long genomic and cDNA sequences for species ranging from yeast to humans. We also analysed other sequences encoding a variety of other proteins as well as regulatory DNA sequences. We discovered that long-range correlations ($\alpha > 1/2$) are characteristic of intron-containing genes and nontranscribed genomic regulatory elements (group A). Thus, the average value (± 2 s.e.m.) of α for the first 14 entries of Table 1 is 0.61 ± 0.03 . By contrast, for cDNA sequences and genes without introns, we find that $\alpha = 1/2$ indicating no long-range correlation (group B). In keeping with this, the average value of α for the last 10 entries of Table 1 is 0.50 ± 0.01 . This significant difference in the value of α for the two groups of nucleotide sequences is confirmed in Fig. 2b,

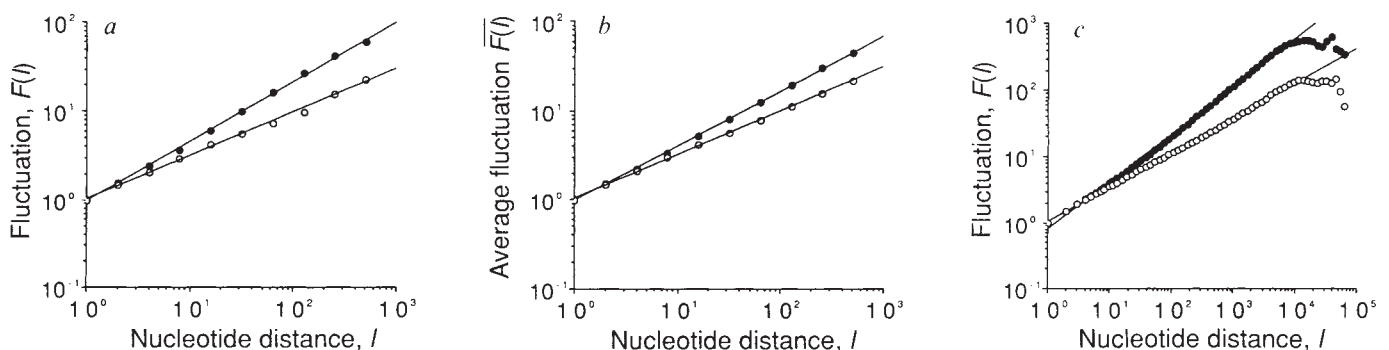


FIG. 2 Double logarithmic plots of a, the mean square fluctuation function $F(l)$ as a function of the linear distance l along the DNA chain for the human β -cardiac myosin heavy chain gene (●, $\alpha \approx 0.67$) and its cDNA (○, $\alpha \approx 0.49$) and b, the average of $F(l)$ over the entries in groups A (●, $\alpha \approx 0.62$) and B (○, $\alpha \approx 0.49$) of Table 1. The difference in the slopes is statistically significant, consistent with the possibility of long-range correlations in group

A and short-range correlations in group B. For clarity, the data points shown are separated by intervals of integer powers of 2. c, A correlation of even longer range for the intron-containing human β -globin chromosomal region (73,376 nucleotides; ●, $\alpha \approx 0.71$). Shown for comparison is the '1-step' standard Markov chain analysis of the same nucleotide sequence, displaying the expected exponent $\alpha = 1/2$ (○, $\alpha \approx 0.52$).

TABLE 1 Summary of the correlation analysis of 24 sequences selected across the phylogenetic spectrum.

Sequence	Code	Comments	Length (nt)	% Introns	α
GROUP A					
Adenovirus type 2	ADBCG	Intron-cont. Virus	35,937	n.d.	0.56
<i>Caenorhabditis elegans</i> MHC gene 1	CELMY01A	Gene	12,241	51	0.61
<i>C. elegans</i> MHC gene 2	CELMY02A	Gene	10,780	44	0.54
<i>C. elegans</i> MHC gene 3	CELMY03A	Gene	11,621	49	0.61
<i>C. elegans</i> MHC unc 54 gene	CELMYUNC	Gene	9,000	25	0.58
Chicken c-myb oncogene	CHKMYB15	Gene (5'-end)	8,200	92	0.60
Chicken embryonic MHC	CHKMYHE	Gene	31,111	78	0.65
<i>Drosophila melanogaster</i> MHC	DROMHC	Gene	22,663	72	0.56
Goat β -globin	GOTGLOBE	Gene*	10,194	n/a	0.58
Human β -globin	HHUMHBB	Chromosomal region	73,326	n/a	0.71
Human metallothionein	HUMMETIA	Gene	2,941	91	0.61
Human α -cardiac MHC	HUMMHCAG1	Gene (N-terminal)	2,366	72	0.65
Human β -cardiac MHC	HUMBMHYH7	Gene	28,438	73	0.67
Rat embryonic skeletal MHC	RATMHC	Gene	25,759	76	0.63
					$\alpha_{\text{mean}} \pm (2 \text{ s.e.m.}) = 0.61 \pm 0.03$
GROUP B					
Bacteriophage λ	LAMCG	Intronless Virus	48,502	0	0.53
Chicken c-myb oncogene	CHKCMYBR	cDNA	2,218	0	0.50
Chicken nonmuscle MHC	CHKMYHN	cDNA	7,003	0	0.47
<i>Dictyostelium discodium</i> MHC	DDIMYHC	cDNA	6,681	0	0.49
<i>Drosophila melanogaster</i> MHC	DROMYONMA	cDNA	6,338	0	0.47
Human β -cardiac MHC	HUMBMHYH7CD	cDNA	6,008	0	0.49
Human dystrophin	HUMDYS	cDNA	13,957	0	0.53
Human embryonic MHC	HUMSMHCE	cDNA (partial)	3,382	0	0.51
Human mitochondrion	HUMMT	Intronless Gene	16,569	0	0.49
Yeast MHC	SCMYO1G [†]	Intronless Gene	6,108	0	0.50
					$\alpha_{\text{mean}} \pm (2 \text{ s.e.m.}) = 0.50 \pm 0.01$

Sequences are divided into two groups on the basis of their intron content; within each group the sequences are ordered alphabetically. Note that $\alpha > 0.5$ implies the existence of long-range correlations, whereas $\alpha \approx 0.5$ implies only short-range correlations. The second column (code) lists the GenBank names (unless specified otherwise).

nt, Number of nucleotides per sequence.

*, ϵ -globin activating region (nontranscribed DNA).

n.d., no data; exon/intron map not fully known.

n/a, not applicable; nontranscribed DNA regions.

MHC, myosin heavy chain.

†, EMBL name.

also find that the only way to obtain a deviation from $\alpha = 1/2$ is to introduce a long-range correlation; for example, studies of an artificial sequence characterized by long-range correlation with correlation parameter of 0.61 yield a graph of the form of Fig. 2a, with a slope of exactly 0.61.

To confirm that the nucleotide correlations are truly long range, we now discuss the breakdown of linearity that must ultimately occur in graphs such as Fig. 2a and b. We do not show the graphs for distances larger than 1,000 because the statistical error increases (not because the correlation vanishes, which would be indicated by the slope changing to the value $\alpha = 1/2$). An increase in statistical error when there are less than roughly 10 independent data sets is usually found for analysis of this sort. For example, if a gene has 10,000 nucleotides, then we obtain only 10 independent sets of data when the calipers are separated by a distance of 1,000. Indeed, the 'fall-off' in the straight line behaviour is typical of all fractal analysis and is also found for artificial sequences of correlated numbers. We find that genes with more nucleotides possess still longer regions of linear (power law) behaviour than the three decades of linearity shown in Fig. 2a and b. For example, Fig. 2c is the correlation graph for the human β -globin chromosomal region, which has 73,376 nucleotides; note that the linearity extends to roughly 7,000 nucleotides. For comparison, Fig. 2c also shows the standard Markov chain analysis, which corroborates the classical result $\alpha = 1/2$.

We have thus introduced a new method to display correlations in the sequence of nucleotides, and defined a quantitative measure of the degree of correlation which is derived from a random walk representation. We find that the nucleotide sequence in intron-containing genes is highly correlated, and that the correlation is remarkably long range, indeed, nucleotides thousands of base pairs distant are correlated. Moreover, the quantitative scaling of the correlation is of the power law form

observed in numerous phenomena having a self-similar or 'fractal' origin. A previous report of long-range correlations in DNA did not provide sufficient evidence to establish this fact⁴. Finally, we note that such long-range correlations are generally associated with the existence of a nonequilibrium dynamic process^{2,5-7}. Interestingly, cDNAs do not have this property and seem to exist in an equilibrium state. Our finding of long-range correlations in intron-containing genes seems to be independent of the particular gene or the encoded protein. It is observed in genes as disparate as myosin heavy chain, β -globin and adenovirus (Table 1). The functional (and structural) role of introns remains uncertain (for review see ref. 8). Although our data do not resolve the 'intron-late'⁹ versus 'intron-early'^{10,11} controversy about gene evolution, they do reveal intriguing fractal properties of genome organization that need to be accounted for by any such theory. □

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1. Tavaré, S. & Giddings, B. W. in *Mathematical Methods for DNA Sequences* (ed. Waterman M. S.) 117-132 (CRC Press, Boca Raton, 1989).
2. Montroll, E. W. & Shlesinger, M. F. in *Nonequilibrium Phenomena II. From Stochastics to Hydrodynamics* (eds Lebowitz, J. L. & Montroll, E. W.) 1-121 (North-Holland, Amsterdam, 1984).
3. Stanley, H. E. *Introduction to Phase Transitions and Critical Phenomena* 120-121 (Oxford University Press, London, 1971).
4. Li, W. *Santa Fe Institute Technical Report No. SFI-91-02* (1991).
5. Dutta, P. & Horn, P. M. *Rev. mod. Phys.* **53**, 497-516 (1981).
6. Bak, P., Tang, C. & Wiesenfeld, K. *Phys. Rev. Lett.* **59**, 381-384 (1987).
7. Shlesinger, M. F. *Rev. phys. Chem.* **39**, 269-290 (1988).
8. Doolittle, W. F. in *Intervening Sequences in Evolution and Development* (eds Stone, E. & Schwartz, R.) 42-62 (Oxford University Press, New York, 1990).
9. Gilbert, W. *Nature* **271**, 501 (1978).
10. Darnell, J. E. Jr *Science* **202**, 1257-1260 (1978).
11. Doolittle, W. F. *Nature* **272**, 581-582 (1978).

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Mutation of protein kinase A causes heterochronic development of *Dictyostelium*

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In heterochronic mutants the relative timing of developmental events is altered compared with the wild type. This generally results in a disordered embryo^{1,2}, though heterochronic mutations may also be an important source of evolutionary variation³. In the rapidly developing (*rde*) mutants of *Dictyostelium*, stalk and spore cells differentiate before morphogenesis is complete. We have traced the lesion in one class of these mutants to the regulatory subunit of cyclic AMP-dependent protein kinase (pk-A). Inactivation of this protein results in the unrestrained activity of the catalytic subunit, so prematurely triggering terminal cell differentiation.

Two complementation groups of *rde* mutants are known in detail⁴⁻⁶. In *rdeA* mutants, aggregation is accelerated and spores differentiate at the base of the stalk instead of the top. In *rdeC* mutants, aggregation is not accelerated but is followed abruptly by the maturation of spore and stalk cells, forming mounds of jumbled cells. Mutant cells developed as submerged monolayers forming spores, whereas wild-type cells remain amoeboid though, significantly, spores can be induced by permeant cAMP analogues⁷. These analogues are believed to bypass the cell surface cAMP receptor and activate pk-A directly. *Dictyostelium* pk-A consists of catalytic and regulatory (C and R) subunits⁸. The inactive (RC) holoenzyme dissociates when cAMP binds to the R subunit to free active C subunits. The enzyme is present at very low levels in growing cells but increases during development⁹.

We screened 17 rapidly developing and sporogenous mutants (mutants selected to form spores in submerged monolayers¹⁰) for the presence of the R subunit. Western blots showed defects in two *rdeC* strains, and no others: HTY506 and HTY510 lacked detectable R subunit (Fig. 1). The third *rdeC* strain, HTY217, had apparently normal levels of the R subunit (see later). Measurement of cAMP-binding activity confirmed this result: crude cell lysates from HTY506 showed no significant binding at any stage of development, whereas HTY217 and its parent NP2 showed the expected developmental increase (Fig. 2).

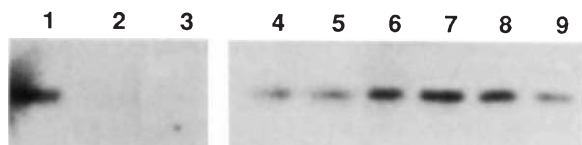


FIG. 1 Western blots of the R subunit in *rde* and sporogenous mutants. Cells were grown on nutrient agar in association with *Klebsiella aerogenes*, washed free of bacteria in 20 mM K_1/K_2PO_4 , 2 mM $MgSO_4$, pH 6.2 and allowed to develop for 7–12 h on agar plates. Frozen pellets of 4×10^7 cells were resuspended in hot (95 °C) gel sample buffer²¹ and boiled for an additional 5 min. Total protein (100 µg) was subjected to acrylamide gel electrophoresis in the presence of 0.1% SDS, blotted onto nitrocellulose filters and immunolabelled with an anti-R antiserum¹¹. Strains were: Lane 1, XP55 12 h (parental); 2, HTY506 12 h (*rdeC*); 3, HTY510 12 h (*rdeC*); 4, HTY217 12 h (*rdeC*); 5, NP2 12 h (parental); 6, HM18, 7 h (sporogenous); 7, HM16 7 h (sporogenous); 8, HM2 7 h (sporogenous); 9, HTY507 9 h (*rdeA*). The other strains analysed (the wild types V12M2 and HM27 and the sporogenous mutants HMs14, 15, 28, 29, 33, 35, 40, 41, 105, 106, 107) gave similar high levels of R subunits as lanes 1 and 4–9 (not shown).

TABLE 1 Assay of pk-A activity from *rdeC* mutants and their parents

	–cAMP	+cAMP	+PKI	+cAMP +PKI
XP55 12 h	0.14	0.29	0.05	0.05
HTY506 12 h	0.15	0.11	0.05	0.03
NP2 16 h	0.05	0.12	0.04	0.02
HTY217 16 h	0.12	0.13	0.04	0.04

The pk-A activity was measured in the presence or absence of 1 mM final cAMP²⁶. To distinguish the pk-A activity from the background protein kinases present in the extract, 20 µM of an inhibitory peptide (PKI) corresponding to residues 5–24 of the bovine heat-stable inhibitor²⁷ was added. This peptide inhibits the *Dictyostelium* C subunit with a K_i of 0.4 µM. The low level of stimulation by cAMP in the parental strain (110% and 140%) is due to partial dissociation of the holoenzyme during preparation of the extracts, as previously described⁹. Thus the total catalytic activity is best computed as the difference between the activity in the presence of cAMP and the activity in the presence of the PKI. By this criterion, the *rdeC* mutants HTY506 and HTY217 both have an active C subunit. Kinase activity is expressed in nmol ^{32}P transferred per min per mg protein.

Northern blots failed to detect any R subunit messenger RNA in HTY506 cells at any time of development tested, but showed the expected expression in its parent (not shown). Southern blots of HTY506 and HTY510 DNA did not show any gross rearrangements of the R subunit gene (not shown), suggesting a point mutation or short deletion in the gene of these strains.

The other *rdeC* allele, which expresses normal levels of the R subunit, was further investigated for a possible defect in the protein. The R subunit gene was cloned from strain HTY217 by polymerase chain reaction (PCR) using primers corresponding to the first 19 and last 18 bases of the coding sequence¹¹. After DNA sequencing, the only alteration found in the mutant was a G → A transition, leading to mutation of alanine 30 to threonine (Fig. 3). This alanine is in the Arg-Arg-Gly-Ala sequence that inhibits the catalytic activity of the C subunit^{12,13}. The mutant R subunit should therefore bind cAMP (as observed) but inhibit poorly the enzymatic activity of the C subunit^{14,15}. As expected for strains with defective R subunits, the protein kinase activity found in *rdeC* mutants was not stimulated by cAMP (Table 1) but could be inhibited by a peptide derived from the specific heat-stable inhibitor of pk-A.

We further confirmed that *rdeC* encodes the R subunit of pk-A by rescue experiments in which HTY217 was transformed with the R subunit cDNA driven by an actin 15 promoter¹⁶. The proportion of cells forming spores in submerged monolayers in the presence of cAMP⁷ was reduced from 50% in HTY217 to

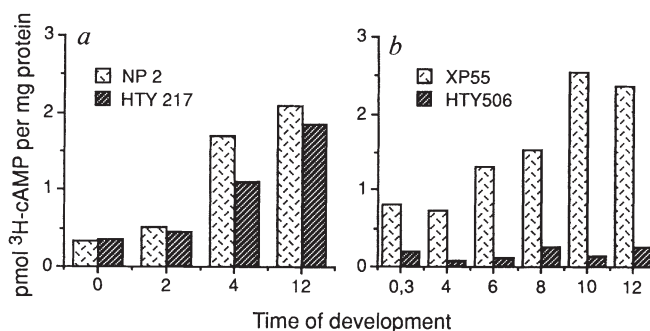


FIG. 2 Binding activity of cAMP in crude extracts. Cells were allowed to develop as described in Fig. 1, aliquots were taken at different times and frozen in liquid nitrogen. The pellets were resuspended in buffer (10 mM MOPS pH 7.5, 4 mM EDTA, 2 mM benzimidazole, 2 mM antipain, 4 mM β -mercaptoethanol, 2 mM phenyl methyl sulphonyl-fluoride, 0.01% triton X100) and centrifuged at 13,000 r.p.m. for 10 min at 4 °C. High-affinity cAMP binding was measured using 0.1 µM 3H -cAMP by the filtration technique as described¹¹. Activities are expressed in pmol cAMP bound per mg of total protein. a, *rdeC* mutant HTY217 and its parent NP2; b, HTY506 compared with its parent XP55. The times of development are indicated.

AA seq.RII/mammals	PHE	ASP	ARG	ARG	VAL	SER	VAL	CYS	ALA	GLU
AA seq.RI/mammals	ARG	ARG	<u>ARG</u>	<u>ARG</u>	GLY	ALA	ILE	SER	ALA	GLU
AA seq.RD/NP2	ARG	LYS	<u>ARG</u>	<u>ARG</u>	GLY	ALA	ILE	SER	SER	GLU
AA seq.RD/HTY217	ARG	LYS	<u>ARG</u>	<u>ARG</u>	GLY	THR	ILE	SER	SER	GLU
DNA R/NP2	CGA	AAA	AGA	AGA	GGT	GCA	ATT	AGT	AGT	GAA
DNA R/HTY217	CGA	AAA	AGA	AGA	GGT	ACA	ATT	AGT	AGT	GAA

FIG. 3 The regulatory subunit of HTY217 is mutated in the sequence essential for inhibition of the activity of the catalytic subunit. The sequence of the pseudo-substrate region from a number of R subunits of pk-A is compared. This region of the protein is believed to bind into the active site of the C subunit and so inhibit its activity. The amino acids (AA) of the consensus sequence are underlined. RI and RII are from the bovine isozyms^{22,23}, RD from *Dictyostelium*, with NP2 being the parental strain and HTY217 the *rdeC* mutant. HTY217 has a G→A transition, resulting in a mutation of Ala to Thr (bold type). PCR amplification²⁴ was made from minipreps of total DNA²⁵. The mutation was detected in two independent experiments by direct sequencing of the PCR products²⁴ from NP2 and HTY217 DNA. The absence of additional mutations was confirmed by sequencing the entire PCR products, after cloning in M13 phage.

less than 1% in its transformant. The transformant also developed at a slower rate and produced a proportion of normal fruiting bodies, though many still showed various degrees of mutant morphology. This incomplete correction of the mutant morphology is probably due to our enforced use of a heterologous promoter to drive R subunit expression, though secondary mutations introduced in the isolation of strain HTY217 may also have contributed.

Because three independent alleles of *rdeC* are affected in the R subunit (two null alleles, one with a point mutation) and the phenotype of an *rdeC* strain can largely be reversed by transformation with a wild-type R subunit-coding region, we conclude that the *rdeC* locus encodes the R subunit of pk-A. As a result of their lack of an effective R subunit, *rdeC* mutants are expected to have a constitutively active C subunit. They are characterized by unrestrained spore formation: mutant prespore cells transform into spores shortly after they appear in normal development and in submerged monolayers they form spores where wild-type cells do not^{7,10}. These results therefore provide strong genetic evidence that spore maturation is normally triggered by the activation of pk-A⁷. Because stalk cell differentiation is also premature in mutant development, it may also be triggered by activation of pk-A. The pk-A is on the signal transduction pathway leading from extracellular cAMP and cAMP levels are known to rise strongly as spores mature during normal development^{5,17}. It therefore seems that the cAMP-signalling pathway ultimately controls spore and, possibly, stalk cell maturation. The cAMP signal transduction pathway bifurcates after the cell surface cAMP receptors with one branch using intracellular cAMP to control terminal cell differentiation, as we have argued. The other branch uses inositol(1,4,5)triphosphate (IP3)/cGMP and is believed to control cell motility and presumably morphogenesis^{18,19}. It is this branching of the second messenger pathways that allows mutants like *rdeC* to be heterochronic, because one branch can be mutated independently of the other. Finally, the very low cAMP levels in *rdeC* mutants⁵ suggests that cAMP levels are under negative feedback control from pk-A. This feedback could also be involved in the adaptation of cells to continuous stimulation by extracellular cAMP²⁰. It is likely that other heterochronic mutants, such as *rdeA*, will also be affected in the cAMP signal-transduction pathway. □

- Abe, K. & Yanagisawa, K. *Dev Biol.* **95**, 200–210 (1983).
- Kessin, R. H. *Cell* **10**, 703–708 (1977).
- Kay, R. R. *Development* **105**, 753–759 (1989).
- de Gunzburg, J., Part, D., Guiso, N. & Veron, M. *Biochemistry* **23**, 3805–3812 (1984).
- de Gunzburg, J. & Veron, M. *EMBO J.* **1**, 1063–1068 (1982).
- Town, C. D., Gross, J. D. & Kay, R. R. *Nature* **262**, 717–719 (1976).
- Mutzel, R., Lacombe, M. L., Simon, M. N., De Gunzburg, J. & Veron, M. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6–10 (1987).
- Taylor, S. S., Buechler, J. A. & Yonemoto, W. A. *Rev. Biochem.* **59**, 971–1005 (1990).
- Knighton, D. R. et al. *Science* **253**, 414–420 (1991).
- Rangel-Aldao, R. & Rosen, O. M. *J. biol. Chem.* **251**, 3375–3380 (1976).
- Kuret, J., Johnson, K. E., Nicolette, C. & Zoller, M. J. *J. biol. Chem.* **251**, 9149–9154 (1988).
- Simon, M. N. et al. *EMBO J.* **8**, 2039–2043 (1989).
- Merkle, R. K., Cooper, K. K. & Rutherford, C. L. *Cell Differ.* **14**, 257–266 (1984).
- Newell, P. C., Europe-Finner, G. N., Small, N. V. & Liu, G. J. *Cell Sci.* **89**, 123–127 (1988).
- Firtel, R. A., van Haastert, P. J. M., Kimmel, A. R. & Devreotes, P. N. *Cell* **58**, 235–239 (1989).
- Devreotes, P. N. & Steck, T. L. *J. Cell Biol.* **80**, 300–309 (1979).
- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Titani, K. et al. *Biochemistry* **23**, 4193–4199 (1984).
- Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A. & Titani, K. *Biochemistry* **23**, 4200–4206 (1984).
- Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. *PCR Protocols* (Academic Press, London, 1990).
- Reymond, C. D. *Nucleic Acids Res.* **15**, 8118 (1987).
- Mutzel, R., Simon, M. N., Lacombe, M. L. & Veron, M. *Biochemistry* **27**, 481–486 (1988).
- Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1613–1616 (1986).

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Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein

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ELUCIDATION of the genetic and molecular basis of plant disease resistance is a major objective in the investigation of plant–microbial interactions. *Xanthomonas campestris* pathovar *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease of pepper and tomato, has been developed as a model host–pathogen system to study the genetic interactions that specify the expression of plant disease resistance^{1–6}. Several plant resistance genes (*Bs1*, *Bs2*, *Bs3*) have been genetically characterized from pepper (*Capsicum annuum*) that determine resistance to particular races of the pathogen carrying specific avirulence genes^{7–9}. For example, pepper plants carrying the resistance locus *Bs3* are resistant to *Xcv* strains expressing the avirulence gene *avrBs3*. Nucleotide sequence analysis of the *avrBs3* gene revealed that the internal portion of the predicted protein product consists of a nearly identical 34 amino acid repeat unit, present in 17.5 copies⁴. We report here that the repetitive region of the *avrBs3* gene determines race-specificity and that deletions of repeat units generate new avirulence specificities and unmask undiscovered resistance genes in pepper and tomato.

Pepper plants harbouring the *Bs3* locus, for example, cultivar ECW-30R, are resistant to *Xcv* race 1 strains. When ECW-30R (*Bs3/Bs3*) is inoculated with *Xcv* race 1, a hypersensitive response¹⁰ is induced and only limited growth of the bacteria is observed in the region of infection^{4,5}. Inoculation of ECW-30R with *Xcv* race 2 gives rise to water-soaked lesions and permits growth of the bacteria to high levels⁴. The genetic locus responsible for induction of a hypersensitive response on ECW-30R is the bacterial avirulence gene *avrBs3*. On the basis of the gene-for-gene hypothesis¹¹ such a race-specific resistance is thought to be the result of the recognition between the product of a particular plant resistance gene and an elicitor that is produced by a corresponding avirulence gene product¹².

The *avrBs3* gene encodes a protein of relative molecular mass 122,000, AvrBs3 (ref. 13). To investigate whether all of the almost identical repeat units in AvrBs3 are necessary for hypersensitive

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- Ambros, V. & Horvitz, H. R. *Science* **226**, 409–416 (1984).
- Ruvkun, G. & Guisto, J. *Nature* **338**, 313–319 (1989).
- Bonner, J. T. *Am. Nat.* **119**, 530–552 (1982).
- Sonneborn, D., White, G. & Sussman, M. *Dev Biol.* **7**, 79–93 (1963).

TABLE 1 Reactions of pepper and tomato caused by *Xcv* wild-type strains and race 2 transconjugants expressing *avrBs3* and representative *avrBs3Δrep* alleles

<i>Xcv</i> strains*	Number of repeats deleted	Pepper		Tomato	Group
		ECW	ECW-30R	Bonny Best	
71-21		S†	R‡	R§	
75-3		R§	R§	S	
85-10		S	S	S	
85-10 (<i>avrBs3</i>)		S	R	S	
85-10 (<i>avrBs3Δrep</i> -109)¶	3	S	R	S	I
85-10 (<i>avrBs3Δrep</i> -110)	3	S	R	S	I
85-10 (<i>avrBs3Δrep</i> -16)	4	R	S	R	II
85-10 (<i>avrBs3Δrep</i> -99)	4	R	S	S	II
85-10 (<i>avrBs3Δrep</i> -15)	6	R	R	R	III
85-10 (<i>avrBs3Δrep</i> -32)	10	R	R	R	III
85-10 (<i>avrBs3Δrep</i> -21)	12	R	R	R	III
85-10 (<i>avrBs3Δrep</i> -14)	1	S	S	S	IV
85-10 (<i>avrBs3Δrep</i> -9)	3	S	S	S	IV
85-10 (<i>avrBs3Δrep</i> -13)	6	S	S	R	IV
85-10 (<i>avrBs3Δrep</i> -111)	9	S	S	R	IV
85-10 (<i>avrBs3Δrep</i> -27)	10	S	S	R	IV
85-10 (<i>avrBs3Δrep</i> -121)	15	S	S	R	IV
85-10 (<i>avrBs3Δrep</i> -45)	17	S	S	S	IV

* Bacteria were inoculated at 10^8 c.f.u. ml⁻¹ as described¹⁵. Only 14 out of 30 *avrBs3Δrep* strains are shown.

† S, susceptible (water-soaked lesion).

‡ R, resistant (hypersensitive response).

§ The hypersensitive response in these interactions is caused by avirulence genes that are not related to *avrBs3*.

|| 85-10 (*avrBs3*) carries the *avrBs3* clone pL3XV1-6 (ref. 4).

¶ 85-10 (*avrBs3Δrep*) are deletion derivatives of *avrBs3*, subcloned into the broad host range vector pLAFR3 (ref. 15) and introduced into strain 85-10 by triparental mating (refs 16, 17).

response induction on ECW-30R, we generated random deletion derivatives differing in the number of repeat units. The restriction enzyme *Bal*I that recognizes one site per repeat unit (102 base pairs (bp)) was used for this purpose. Plasmids containing the deletion derivatives (*avrBs3Δrep*) were introduced into a *Xcv* race 2 strain. In all *avrBs3Δrep* strains a protein of the expected size could be identified by western blotting (data not shown). According to the induced plant reactions on pepper the

avrBs3Δrep mutants were classified into four groups, representatives of which are shown in Table 1. Group 1 *avrBs3Δrep* mutants retained the ability to induce a hypersensitive response on ECW-30R whereas most mutants tested (24 out of 30; group II+IV) had lost this ability. When tested on ECW which is nearly isogenic to ECW-30R, differing only at the *Bs3* locus, some *avrBs3Δrep* mutants could induce a hypersensitive response (group II). The group III *avrBs3Δrep* mutants caused

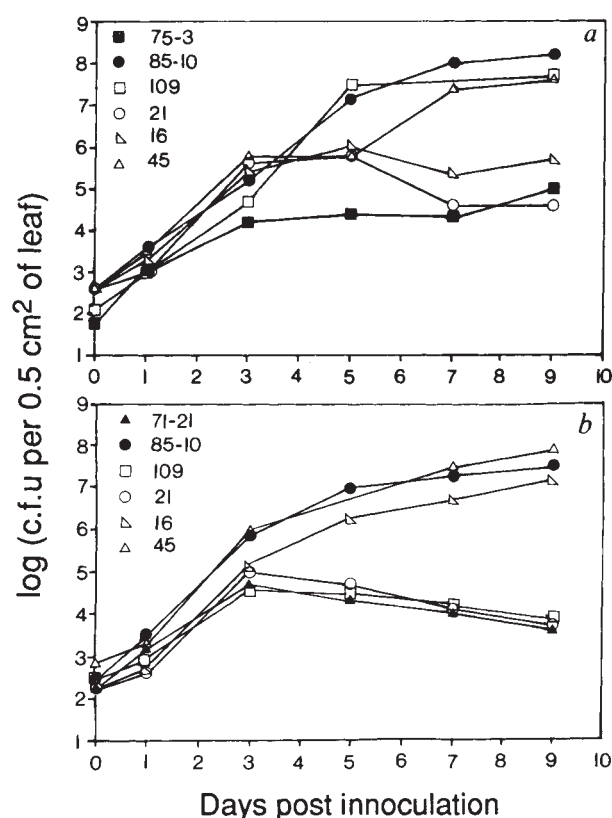


FIG. 1 Time course of multiplication of wild-type *Xcv* strains and transconjugants in ECW (a) and ECW-30R (b) pepper cultivars. The following *Xcv* strains were used: race 1 strain 71-21 (source of *avrBs3*), race 2 strain 85-10, tomato strain 75-3 and strain 85-10 transconjugants carrying *avrBs3Δrep* derivatives Δ rep-109 (group I), Δ rep-21 (group III), Δ rep-16 (group II) and Δ rep-45 (group IV) (see also Table 1 for phenotypic reactions). METHODS. Bacteria were inoculated into pepper leaves at 5×10^4 c.f.u. ml⁻¹ in 1 mM MgCl₂ as described in ref. 15. Values are the means of three samples taken at each time point. For description of the deletion derivatives see Fig. 2.

an intermediate reaction, between hypersensitive response and water-soaked lesions, on both ECW and ECW-30R.

To determine how the hypersensitive response or water-soaked lesion phenotype correlated with the *in planta* growth of the respective bacteria, we followed the multiplication of one representative strain of each group in both pepper cultivars (Fig. 1). Those *avrBs3Δrep* strains that were associated with water-soaked lesions multiplied to a high density differing from that of strain 85-10 by less than a factor of 2. The hypersensitive response inducing wild-type strains 71-21 (on ECW-30R) and 75-3 (on ECW), as well as hypersensitive response-inducing *avrBs3Δrep* strains, including strain carrying *avrBs3Δrep-21* (intermediate hypersensitive response), multiplied as expected; after 9 days their density was at least two orders of magnitude lower than that of strain 85-10. These results prove that the *avrBs3* deletion derivatives that induce a novel hypersensitive response on pepper are functional alleles of this avirulence gene.

Two putative plant resistance loci recognized by different *avrBs3Δrep* alleles were characterized genetically by inoculation of F_2 progeny (332 plants) from a cross between ECW and ECW-30R (*Bs3/Bs3*, *Bs3/bs3*, *bs3/bs3*; 1:2:1 segregation) (Table 2). Derivative *avrBs3Δrep-109* induced a hypersensitive response only on plants that were either homozygous or heterozygous for *Bs3*, whereas water-soaked lesions occurred on *bs3/bs3* plants. This suggests that derivative *avrBs3Δrep-109* recognizes *Bs3* or a dominant locus closely linked to *Bs3*. The inoculation tests of mutant *avrBs3Δrep-16* on the F_2 progeny plants indicate that the plant resistance locus recognized might be identical or linked to *bs3*. The former possibility is intriguing because the *bs3* allele in ECW and in heterozygous *Bs3/bs3* plants might behave like a functional, dominant resistance allele as does *Bs3* with *avrBs3*.

To analyse whether resistance determinants corresponding to *avrBs3* deletion derivatives can be unmasked in a different plant species as well, all transconjugants were tested for their phenotype on tomato cultivar Bonny Best. Surprisingly, most of the different *avrBs3Δrep* strains caused a hypersensitive response on this tomato cultivar. Among these were 16 *avrBs3Δrep* derivatives that were non-functional on pepper (Table 1).

What is the distinctive feature of the *avrBs3Δrep* alleles that gives rise to their avirulence specificity? Because a number of deletion derivatives, despite being of identical size, belong to different reaction groups, the length of the repetitive region cannot be the relevant parameter for the function of these genes.

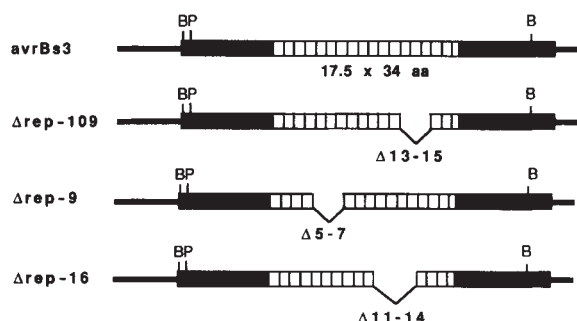


FIG. 2 Overall structure of the *avrBs3* gene and sequenced deletion derivatives. The thick bar indicates the ORF in pUXV1006 that expresses AvrBs3 (refs 4, 13). Each repeat unit (102 bp) is represented by an open box. The DNA sequences of the derivatives $\Delta rep-109$, 9 and 16 were identical to the *avrBs3* sequence⁴ except for the deletion of repeat units at the positions indicated. B, BamHI site; P, PstI site.

METHODS. The deletion derivatives of *avrBs3* were generated by partial digestion of pUXV1006 with *BalI* that recognizes one site per repeat unit. After size fractionation the DNA was religated and transformed into *E. coli* DH5 α . The sequence was determined for the entire gene using *avrBs3*-specific primers for the unique regions and unidirectional deletion clones for the repetitive region as described in ref. 4.

TABLE 2 Reactions of a segregating F_2 population of an ECW-30R $\times$ ECW cross

Genotype	Number of F_2 plants	<i>avrBs3</i> allele in <i>Xcv</i> 85-10		
		<i>avrBs3</i>	$\Delta rep-109$	$\Delta rep-16$
<i>Bs3/Bs3</i> *	72	R	R	S
<i>Bs3/bs3</i>	163	R	R	R
<i>bs3/bs3</i>	97	S	S	R

A heterozygous *Bs3/bs3* plant from a cross of the nearly isogenic pepper cultivars ECW-30R (*Bs3/Bs3*) $\times$ ECW (*bs3/bs3*) was selfed. A total of 332 plants segregating for *Bs3* were analysed. The strains used are described in Table 1. Bacteria were inoculated at 10^8 c.f.u. ml⁻¹ as described¹⁵. S, susceptible; R, resistant (hypersensitive response).

* The (*Bs3/Bs3*) genotype was confirmed by testing 14 populations (about 15 plants each) of different selfed F_2 plants that were susceptible for $\Delta rep-16$.

For three derivatives the sequence has been determined (Fig. 2). The sequences are identical to the original *avrBs3* except for the length of the repetitive region. In each derivative the deletion occurred at a different position. The distinct functions of *avrBs3Δrep-9* (non-functional on pepper) and *avrBs3Δrep-109* (hypersensitive response on ECW-30R) must therefore be due to the position of a repeat in the repetitive region and the type(s) of repeat units present or absent. This is also true for *avrBs3Δrep-16* which has gained a new specificity. Thus a single or a few amino acid differences in the internal region of the protein determine its avirulence function.

The finding of unknown resistance determinants in pepper and tomato leads us to speculate that plant resistance genes complementary to *avrBs3* and its alleles may be widespread in the plant kingdom. Reciprocally, proteins homologous to AvrBs3 that may have an avirulence function occur naturally in certain strains of *Xcv* and in different pathovars of *Xanthomonas* (ref. 13 and I. Balbo, J.C.-S. and U.B., manuscript in preparation). This suggests that *avrBs3* may be one member of a family of homologous avirulence genes.

To account for the great diversity of avirulence specificity we favour a direct interaction between the avirulence gene product and the corresponding resistance gene product, possibly mediated by the repetitive structure of the *avrBs3* protein and its allelic derivatives. This model is supported by the observation of intermediate hypersensitive response that could be due to a weaker binding of the molecules involved. The only example for a postulated direct interaction of an avirulence gene product with the plant is the fungal avirulence gene *avr9* from *Cladosporium fulvum*¹⁴.

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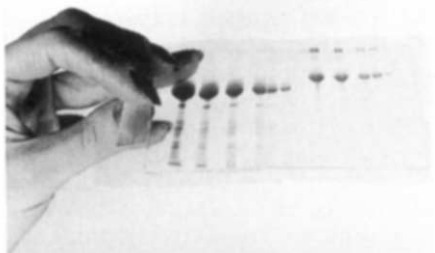
- Swanson, J., Kearney, B., Dahlbeck, D. & Staskawicz, B. J. *Molec. Plant-Microbe Interact.* **1**, 5-9 (1988).
- Ronald, P. C. & Staskawicz, B. J. *Molec. Plant-Microbe Interact.* **1**, 191-198 (1988).
- Kearney, B., Ronald, P. C., Dahlbeck, D. & Staskawicz, B. J. *Nature* **322**, 541-543 (1988).
- Bonas, U., Stall, R. E. & Staskawicz, B. J. *Molec. gen. Genet.* **218**, 127-136 (1989).
- Minsavage, G. V. et al. *Molec. Plant-Microbe Interact.* **3**, 41-47 (1990).
- Kearney, B. & Staskawicz, B. J. *Nature* **346**, 385-386 (1990).
- Cook, A. A. & Guevara, Y. G. *Pl. Dis.* **68**, 329-330 (1984).
- Kim, B. S. & Hartman, R. W. *Pl. Dis.* **69**, 233-235 (1985).
- Hibberd, A. M., Stall, R. E. & Bassett, M. J. *Phytopathology* **77**, 1304-1307 (1987).
- Klement, Z. in *Phytopathogenic Prokaryotes* Vol. 2 (eds Mount, M. S. & Lacy, G. H.) 149-177 (Academic, New York, 1982).
- Flor, H. H. *Phytopathology* **45**, 680-685 (1955).
- Albersheim, P. & Anderson-Prouty, A. J. *Rev. Pl. Physiol.* **26**, 31-52 (1975).
- Knoop, V., Staskawicz, B. J. & Bonas, U. *J. Bact.* **173**, 7142-7150 (1991).
- Scholtens-Toma, I. M. J. & de Wit, G. J. M. *Physiol. molec. Pl. Pathol.* **33**, 59-67 (1988).
- Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. *J. Bact.* **169**, 5789-5794 (1987).
- Figurski, D. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1648-1652 (1979).
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7347-7351 (1980).

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Molecular tools of the trade

New products for molecular biologists include software for microsatellite analysis, a kit for the direct labelling of Fab', IgG or other proteins and molecules, and chromosome paints for the identification of individual human chromosomes.

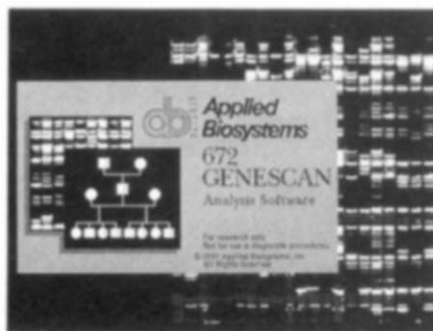
BioGelWrap from BioDesign is the new quick and easy way to **dry and store all types of polyacrylamide and agarose gels** (*Reader Service No. 100*). The unique pore configuration of BioGelWrap allows water in a gel to be readily evaporated directly through the BioGelWrap membrane, eliminating the need for a vacuum hot-plate gel dryer. The membrane bonds to both the gel surface and itself, so that the dried gel is sealed between two layers of tough, transparent and stable membrane without wrinkling or cracking. In this form, the gel can be stored permanently, photographed, examined by densitometry, or shown on an overhead projector. Stored in this way,



No need for a gel dryer with BioGelWrap.

BioDesign claims that the colour of stains, such as silver or brilliant blue, remains stable for years. The BioGelWrap drying system can be used on all types of electrophoretic and immunodiffusion gels.

To meet the demand for quantitative methods for DNA fingerprinting and linkage studies, Applied Biosystems has introduced new **GENESCAN software for microsatellite analysis** (*Reader Service No. 101*). Microsatellites, also known as simple sequence tandem repeat polymorphisms, are fast becoming the markers of choice for linkage mapping of genes. These new markers are more numerous and polymorphic and are, therefore, far easier to find than classical restriction fragment length polymorphisms. Microsatellites show differences in the number of tandemly repeated sequences. And, these differences, also called alleles, are recognized by GENESCAN as fluorescence-labelled polymerase chain reaction (PCR) products. GENESCAN, which is designed for use with the 373A DNA sequencer, detects and sizes microsatellites in the 50–350-base pair range with greater than 99 per cent precision, detecting as little as 100 attomoles of sample, ABI says. As GENESCAN distinguishes among four fluorescent labels, many assays can be done in a single PCR reaction



GENESCAN — ABI's genetic analysis software.

tube, and many independent loci can be run with a size standard in a single gel lane. This, ABI says, saves time and reagent costs. Flexible on-screen viewing and a wide linear dynamic range of detection are intended to make data editing and interpretation easy, even when a large variation in DNA concentration exists between bands.

Extracting with ease

Boehringer has developed a new product called Agarase that is designed to make the routine molecular biology procedure of **isolating DNA fragments from agarose gels** quick and easy to perform (*Reader Service No. 102*). Agarase digests low-melting-point agarose to produce a clear solution of 'neoagar-oligosaccharides'. After a one-hour incubation at 45 °C in standard Tris-acetate-EDTA or Tris-borate-EDTA buffer, DNA can be recovered by ethanol precipitation — the products of digestion being soluble in alcohol. DNA in the digested agarose solution can also be directly manipulated, for example, by restriction enzyme digestion or ligation.

MicroProbe announces the availability of the IsoQuick nucleic acid extraction Kit, a procedure for the **direct extraction and purification of DNA or total nucleic acid**, designed especially for use with the polymerase chain reaction or restriction enzyme digestion (*Reader Service No. 103*). Using a simple phase-separation technique, the new kit allows researchers to extract and purify DNA or total nucleic acid from whole blood, Gram-negative bacteria, cultured mammalian cells and other complex biological samples, without the need for reagents such as phenol or chloroform. According to MicroProbe, researchers can extract purified DNA using a three-step procedure in 20 minutes. Samples are lysed with guanidine thiocyanate

(GuSCN), which both disrupts cellular integrity and inhibits nuclease (DNase and RNase) activity, while stabilizing the nucleic acid in the lysate. The GuSCN lysate is then mixed with a non-corrosive extraction reagent containing a nuclease-binding matrix. The aqueous and organic phases are separated by centrifugation with the nucleic acid partitioning into the aqueous phase, while protein and other cellular contaminants remain in the organic phase. The extracted nucleic acid is precipitated with alcohol and can be dissolved in either RNase-free water or an appropriate buffer. An optional 35 minute extraction procedure for total nucleic acid provides the researcher with DNA and RNA of a purity and yield comparable to that obtained by the phenol/chloroform method. The kit has sufficient reagents for up to 100 extractions (100 µl sample volume).

Brewers of the Japanese rice wine, sake, have stumbled across an **enzyme that has the ability to break down yeast cells**. This enzyme, known as Zymolyase 100T, is being made available to researchers by ICN Flow (*Reader Service No. 104*). Isolated from the bacteria *Arthrobacter luteus*, the enzyme lyses (or breaks down) the cell walls of viable yeast cells, enabling DNA insertion and manipulation. Zymolyase 100T should find application in a wide range of disciplines, including genetics, molecular biology, immunology, biochemistry, protein and cancer research. It is available as a powder at two levels of activity — 100,000 and 20,000 units per gram. A 1-g vial of Zymolyase 100T at the lower level of activity costs £144.75 (UK).

Assorted kits

The TimeSaver cDNA synthesis kit from Pharmacia P-L Biochemicals is designed to allow **cDNA libraries to be prepared from mRNA** in a day (*Reader Service*



cDNA synthesis from mRNA in a day.

No. 105). The kit includes Oligo(dT) 12–18 and random hexamers [pd(N)6] for priming cDNA synthesis and *EcoR* I/*Not* I cohesive-end adaptors to facilitate cloning. Pharmacia claims that the considerable time savings in producing representative libraries containing full-length cDNAs using a primer of choice is made possible by the specially developed rapid ligation procedure and the use of SizeSep 400 Spun Columns for intermediate purifications. When used in conjunction with the company's directional cloning toolbox, the TimeSaver cDNA synthesis kit can synthesize cDNA with asymmetric ends for unidirectional cloning.

Ambion announces its new group of MEGAscript *in vitro* transcription kits (SP6, T7 or T3 RNA polymerase) for the large-scale synthesis of RNA (Reader Service No. 106). With these kits, Ambion claims that at least 10 times the amount of RNA can be synthesized as in conventional *in vitro* transcription reactions. Stated uses include synthesizing RNA for *in vitro* translation, micro-injection, isolation of RNA binding proteins, subtractive cDNA library construction and ribozyme studies. The development of cloning vectors containing promoters for SP6, T7 and T3 RNA polymerases has made the *in vitro* synthesis of single-stranded RNA molecules a routine laboratory procedure. *In vitro* transcription reactions are used to synthesize both small mass amounts of high-specific-activity RNA probes and larger amounts of unlabelled RNA. Under standard conditions, the synthesis of 5–10 µg of unlabelled RNA in a 20-µl reaction can routinely be obtained. The company says that it has developed reaction conditions that allow much higher levels of nucleotides to be used than in conventional transcription reactions. This results in substantial increases in the yield of transcription products. MEGAscript *in vitro* transcription kits contain sufficient reagents to perform forty 20-µl reactions. The complete kit contains nucleotides, reaction buffer, enzymes, placental

ribonuclease inhibitor, RNase-free DNase and control template.

United States Biochemical is offering a Δ Taq cycle sequencing kit based on Δ Taq DNA polymerase, which is recommended for use when only very small quantities of template DNA are available (Reader Service No. 107). Cycle sequencing takes advantage of the thermal stability of Taq DNA polymerase to anneal repeatedly (by thermal cycling) a labelled primer to the template and extend it, terminating with the incorporation of a dideoxynucleotide. In this way, a relatively large amount of dideoxy-terminated DNA can be produced using only a small amount of template DNA. USB has developed a new cycle sequencing method using incorporation of labelled nucleotide instead of using labelled primer. Thus, USB says, it is equally effective with ^{35}S or ^{32}P and requires only the normal sequencing label, α - ^{35}S dATP, α - ^{32}P dATP, α - ^{35}S dCTP or α - ^{32}P dCTP. The protocol involves a labelling and a termination step. The labelling step (which is also thermally cycled) is run using only three of the four dNTPs present. This limits extensions to a fixed length, stopping for want of the fourth nucleotide. Careful choice of the labelled nucleotide and the 'missing' nucleotide is designed to ensure that a maximum number of labelled nucleotides are incorporated into the extended primer during this step. After a sufficient number of cycles, the reaction mixture is divided among four termination reactions, each containing a different dideoxynucleotide. The termination reaction mixtures are then thermally cycled, stop solution added, and the products loaded onto a sequencing gel. Sequencing using this method is effective for both single-stranded M13 DNA and for double-stranded plasmid DNAs. The kit costs \$185 (US).

Promega has introduced two rabbit reticulocyte lysate systems—TnT lysate systems—that can be used to produce

proteins *in vitro* directly from coding sequence DNA cloned downstream of T7 or SP6 RNA polymerase promoters (Reader Service No. 108). The company says that the single-tube procedure eliminates the time and effort required to prepare RNA for a standard lysate translation. When starting with 1 µg circular plasmid DNA, *in vitro* translation results can visually be seen by autoradiography in 5–6 hours. In the SP6 system, TnT lysate system reactions can produce 2–6 times the amount of protein of a standard lysate reaction, thus reducing film exposure times and increasing the amount of protein available for functional studies. The TnT lysate systems should be particularly useful for the rapid screening and analysis of mutants. A non-radioactive, functional luciferase assay is included as a control. A protocol and sufficient reagents for thirty 50-µl translation reactions are included.

The new digoxigenin *in situ* hybridization detection kit from British Bio-technology is designed for the detection of digoxigenin-labelled oligonucleotides that have been annealed to target mRNA by *in situ* hybridization (Reader Service No. 109). Once hybridized to target mRNA, the labelled oligonucleotides are detected using a specific sheep Fab fragment conjugated directly to alkaline phosphatase. BBT says the digoxigenin system for non-radioactive labelling and detection offers several benefits over other labelling methods. Digoxigenin, unlike biotin, does not occur naturally in animal tissues and, therefore, provides a good signal-to-noise ratio. In addition, digoxigenin-labelled probes are stable and offer good cellular resolution typical of non-radioactive *in situ* hybridization. Results can be achieved in about two days.

Labelling update

Recently, Nanoprobes introduced a 1.4-nm Nanogold-Fab' antibody probe. Nanogold is not colloidal gold but a gold compound covalently attached to the antibody. The company is now introducing a Nanogold labelling kit that allows researchers to label covalently Fab', IgG or other molecules directly with the 1.4-nm Nanogold (Reader Service No. 110). The kit should be of interest in a wide range of fields and applications and is intended to provide primary detection that is more sensitive than fluorescence or radioactivity. According to Nanoprobes, the gold probe, Nanogold-Fab', offers superior penetration into cells, tissue blocks and nuclei over colloidal gold probes. In addition, the company claims that unlike colloidal gold, Nanogold is not 'sticky'; no aggregates form and background binding is reduced. And, the covalent attachment of the gold provides good stability over time, ranges of pH and ionic strength where colloidal gold probes dissociate.

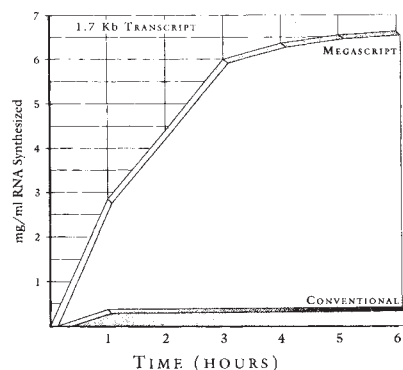


FIGURE 1. 20 µl transcription reactions containing 1 µg template DNA (1.7 Kb transcript size), 40 U of T7 RNA polymerase, buffer, NTPs and ^{32}P -UTP were incubated at 37°. 1 µl aliquots were removed at 1 hour intervals and acid precipitable cpm determined by scintillation counting.

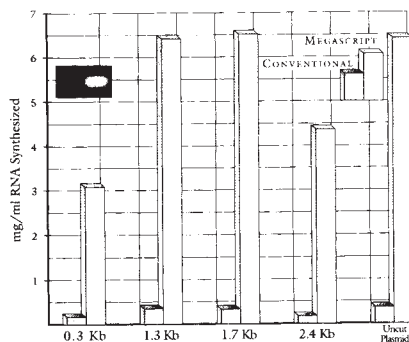
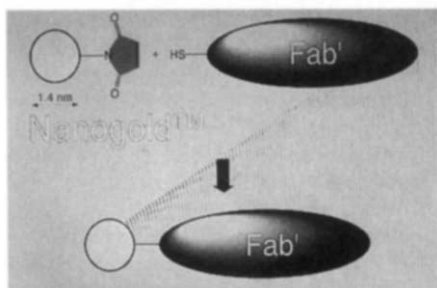


FIGURE 2. Transcription reactions were as described in Figure 1 with 1 µg of different DNA templates coding for the indicated sized transcripts added to each reaction. Inset shows 0.25 µl aliquots of both conventional and MEGAscript reactions transcribing a 0.3 Kb transcript separated on a 2% agarose gel and ethidium bromide stained.

Comparison of MEGAscript with conventional *in vitro* transcription reactions.



Nanogold — Nanoprobes' gold standard.

The Nanogold is supplied as monomaleimido-Nanogold (which reacts with free sulfhydryls) and is mixed with Fab' or protein to be labelled. After two hours, the Nanogold-protein product is purified on a gel filtration column and is then ready for use. Nanogold develops with silver developer, which 'grows' the gold into silver grains whose size depends on the development time. This, Nanoprobes says, makes these probes useful for electron microscopy, and light and confocal microscopy. With further development (of 25 minutes), they can be used in immunoblots or western blots for the sensitive detection of 0.1 pg of antigen.

Enzo's new BIOPROBE random priming kit, developed using the company's proprietary non-radioactive labelling technology, can be used for the **biotin-labelling of DNA probes** (Reader Service No. 111). The procedure is based on the annealing of random sequence hexanucleotide primers to the DNA to be labelled. Klenow enzyme then catalyses the sequential addition of nucleotide residues to the 3'-hydroxyl terminus of the hexanucleotide primers, resulting in a net synthesis of DNA. In the presence of Enzo's patented nucleotide, Bio-11-dUTP, the synthesized DNA will be labelled with biotin. Random-primed synthesis is a template-dependent reaction and, therefore, the incorporation of Bio-11-dUTP is determined by the A-T composition of the DNA. The biotinylated DNA probes can be used for a variety of filter hybridization procedures, including Southern, northern and dot-blot analyses,

and *in situ* procedures. The hybridized biotinylated DNA probe can then be detected with an appropriate streptavidin-biotin detection complex.

Reagent round-up

Genosys Biotechnologies is now offering a host of **custom synthesis services** for the molecular biologist (Reader Service No. 112). In addition to desalted or purified oligonucleotides, custom services offered by Genosys include the synthesis of DNA with modified backbones (for example, phosphorothioates), 5' and 3' modifications such as biotinylation, phosphorylation and fluorescein, and the incorporation of modified bases. Microgram to gram quantities can be produced. The company has also moved into the area of gene synthesis and can provide custom genes that are assembled, cloned, sequence verified and ready for use.

Cambio's **chromosome painting kit** is designed to provide a highly specific method for the identification of individual human chromosomes (Reader Service No. 113). The painting kit enables the detection of translocations and insertions on metaphase chromosomes; marker chromosomes may also be identified and complex karyotypes can be analysed. Cambio says the paints are highly chromosome-specific and provide even coverage of chromosome arms, unlike paints based on cloned libraries. The paints can visually be seen with the biotin/avidin system, which allows use of any fluorochrome coupled to avidin or streptavidin. Cambio paints are available for the following human chromosomes: 1 to 9, 13 to 22 and X and Y, individually or in any combination. The kits contain chromosome counterstains, which provide fluorescence reverse banding simultaneously with a chromosome-specific signal. These are compatible with both epifluorescence and laser-scanning confocal microscopy.

For improved transformation efficiencies over chemical methods, Invitrogen recommends its new line of ready-to-use

electrocompetent *Escherichia coli* (Reader Service No. 114). With these **electrocompetent cells**, Invitrogen says it is possible to introduce nanogram amounts of DNA into cells to increase efficiencies up to 10 fold. A wide variety of electrocompetent cells are available, which are recombination and methylation deficient and allow blue/white screening to ensure success in applications where efficient transformation is essential.

PROTORGOLD is a new colloidal gold solution from BioCell Research Laboratories designed for the sensitive **staining of proteins immobilized onto membranes** by dot blots or by western blots from electrophoretic gels (Reader Service



Protein staining for western blots.

No. 115). The gold particles are negatively charged and bind selectively to the blotted proteins with low background, the company says. During a one-step incubation process, proteins stain red in minutes through the accumulation of gold particles; optimum staining is reached after two hours, although BioCell adds that staining can be continued indefinitely without the danger of overstaining. The stated sensitivity is better than one picogram for bovine serum albumin dot-blotted onto a nitrocellulose membrane. Enhancement of the signal with the BioCell silver enhancing kit provides a further 10–100-times sensitivity.

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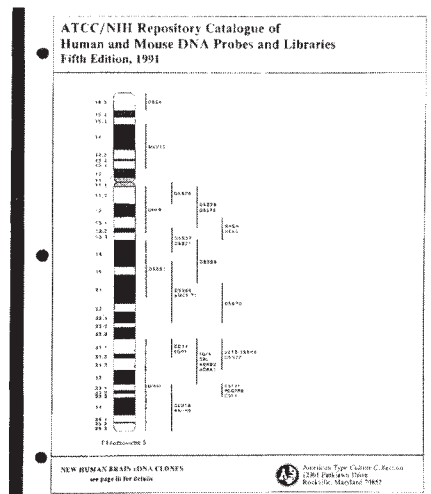
Reader Service No.17

stant visualization of protein separation during electrophoresis (*Reader Service No. 116*). The Rainbow Markers run as discrete coloured bands of similar intensities to allow the protein separation to be followed while electrophoresis is still in progress. The markers are available as non-radioactive and ^{14}C -methylated standards. Visually, Rainbow Markers provide a check of efficient membrane transfer, with the methylated markers providing positive identification in subsequent autoradiography. Two molecular-weight ranges are available: 2.3kD to 46 kD and 14.3 kD to 200 kD. Each is supplied in 50 per cent glycerol with sufficient material for 25 applications.

International Laboratory Services has added three new **lambda DNA size markers** to its existing range, including a *Hind* III, an *Eco*R I and a combined *Eco*R I/*Hind* III restriction endonuclease digest (*Reader Service No. 117*). These yield fragment sizes ranging from 23130–125, 21226–3530 and 21226–125 base pairs, respectively, giving clearly defined bands and enabling easy estimation of the size of the sample DNA when run on an agarose gel. The markers are supplied in a loading buffer containing bromophenol blue and glycerol, and are supplied at 1 µg per 5 µl in 50-µg pack sizes.

Shelf stockers

Over 2,600 human and mouse DNA probes and libraries available from the American Type Culture Collection are

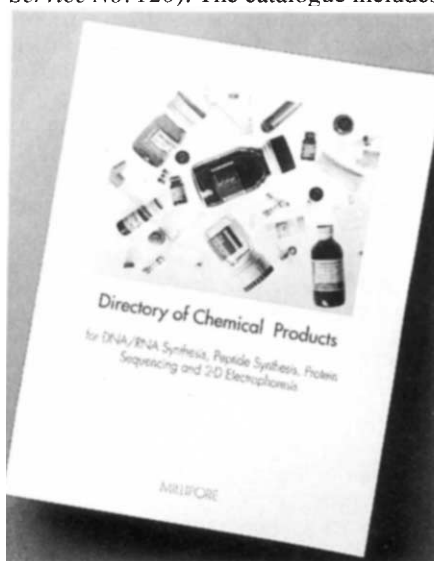


ATCC/NIH repository catalogue of human and mouse DNA probes and libraries.

listed in the organization's (5th edition) **ATCC/NIH repository catalogue of human and mouse DNA probes and libraries** (*Reader Service No. 118*). The new catalogue features the initial deposits of clones of human brain cDNAs being sequenced by the laboratory of J. Craig Venter (National Institutes of Health's National Institute of Neurological Disorders and Stroke) as part of a project to identify genes expressed in the human brain (see *Science* **252**, 1651–1656; 1991). The catalogue also lists oligonucleotide primers, oncogene/transforming protein probes and clones, and bacterial hosts for transformation or plating libraries available from ATCC.

The 1992 Gibco BRL **catalogue and reference guide** — the first comprehensive company catalogue issued since 1990 — is now available free on request (*Reader Service No. 119*). This latest edition includes several helpful changes that make it more user-friendly. Chapters have been reorganized and, in some cases, renamed, to be more orientated towards applications. There is also more extensive cross-referencing, particularly in the cell culture sections. Over 600 new products for cell culture, molecular biology, cell biology, immunology and related apparatus are now all located within one volume. The expanded reference guide now includes a subject index for FOCUS, the quarterly life science magazine published by Life Technologies. The subject index, comparative charts, troubleshooting sections, restriction maps and other reference features should make the 1992 catalogue and reference guide a handy laboratory resource.

Chemicals for DNA and RNA synthesis, peptide synthesis, protein sequencing and two-dimensional electrophoresis are featured in the 72-page **bioscience chemicals catalogue** from Millipore (*Reader Service No. 120*). The catalogue includes



Bioscience chemicals catalogue monomers and amino acids, columns and bulk supports, kits, reagents and ancillary supplies for Millipore and other brands of instrumentation. Each section includes a chemistry overview with illustrations and descriptions of the reactions taking place. Other features include a thumb index by technology, an alphabetical index, a glossary, technical tables and structural formulae.

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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